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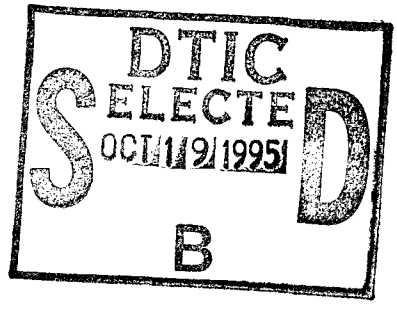

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13. ABSTRACT *(Maximum 200 words)*

We have developed techniques that allow the analysis of multiple chromosomal loci from single paraffin sections from breast cancer lesions. This approach is being used to allelotype small preinvasive breast cancer lesions, correlating with other parameters such as specific gene mutations and expression and with interphase cytogenetics on the same lesions. We identified the chromosome arms most frequently affected by allelic losses and imbalances at preinvasive stages of breast carcinogenesis and those allelic losses involved in more advanced stages of progression. We are continuing with these studies and further refining the mapping of specific subchromosome areas involved in early breast tumorigenesis. In the course of these studies we observed that microsatellite instability (replication error phenotype, RER^+) is characteristic of a subset of breast tumors (invasive lobular breast carcinomas), indicating the possible involvement of defective DNA mismatch repair genes in these tumors. Studies are ongoing to address this point and to evaluate the use of RER phenotype determination as a prognostic-diagnostic tool.

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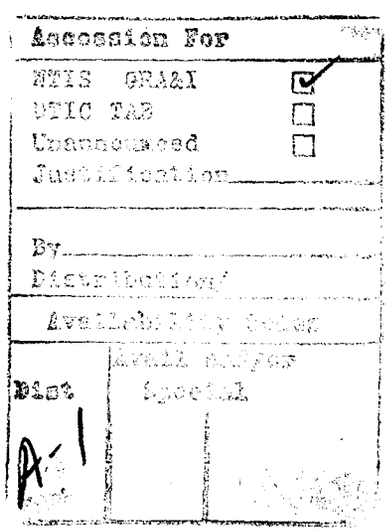

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INTRODUCTION

Numerous studies have focused on the role of gene and chromosome abnormalities in human breast cancer, but to date no clear model of critical events or delineation of primary abnormalities has emerged (Thompson, et al., 1993; Trent, et al., 1993; reviewed in Devilee and Cornelisse, 1994). Specific somatic mutations, altered receptor levels, chromosomal structural or numerical changes, and specific allelic losses have been observed, with frequencies varying from study to study (reviewed in Devilee and Cornelisse, 1994). These include amplification or overexpression of oncogenes and growth factors such as ERBB2, MYC, EGFR, FGF3, and cyclin genes D and E (reviewed in Devilee and Cornelisse, 1994), changes in presence and function in cell surface receptors such as ER and PR, P53 mutations and allelic losses involving chromosomal regions 1p34-p35, 1q23-q32, 3p21-p25, 6q, 7q31, 11p15, 13q14, 16q, 17p13, 17q, 18q23-qter and 22q (reviewed in Devilee and Cornelisse, 1994). Recently the breast cancer susceptibility genes, BRCA1 and BRCA2 have been identified. Their mutation was shown to be a major cause of inherited susceptibility in early-onset disease (Miki, et al., 1994), but this accounts for only approximately 4-5% of breast cancers. BRCA1 appears not to be commonly involved in sporadic forms of breast cancer (Futreal, et al., 1994).

The relevance and role of most of the described genetic abnormalities in the more common 'sporadic' forms of breast cancer are very unclear even though the research effort has been considerable. In fact, the picture is quite confusing and uncertain with numerous mutations described and with several discrepancies among different studies. An obvious reason for such a complex scenario is the inter and intratumor heterogeneity characteristic of breast cancer. In addition, most of the cytogenetic and molecular information on breast cancer have been obtained from the analysis of advanced invasive carcinomas and metastases. **One of the most important issues is to determine whether specific mutations play a relevant role as causative factors or are simply the consequence of the tumor genomic instability.**

Our hypothesis is that a high level of genomic instability already exists at preinvasive stages of breast cancer development. *One of the major objectives of this project is to identify a set of molecular tools that can be used as reliable indicators to identify those preinvasive lesions with higher level of genomic instability and as a consequence worse prognosis.*

To that end the studies described in the original application focused on preinvasive and early stages of breast cancer development in order to identify the earliest detectable allelic and chromosomal abnormalities correlating with the histological grading and subtype of the lesions. The aim is to determine not only the timing for presentation of specific anomalies as well as determining the general level of genomic instability at define preinvasive stages.

Part of the specific goals of this project are to generate a thorough allelotypic study at early stages of breast cancer development (preinvasive stages), correlating the findings at the molecular level with the interphase cytogenetic profile of the same lesions and in the future with other diagnostic prognostic indicators.

These studies will provide information of relevance for understanding mechanisms of breast cancer development as well as possibly developing tools of prognostic-diagnostic significance.

BODY

Technical approach for performing breast cancer genotype-phenotype studies from paraffin embedded tissue sections.

The best obvious source of material for the identification of the various stages of progression is available from paraffin-embedded tissues used in routine diagnostic procedures and that can be obtained from pathology archives. **A first phase** of this project consisted in the optimization of a comprehensive technical approach for allowing a multiparametric analysis of human breast cancer lesions from paraffin-embedded tissue sections. The techniques developed and representative results obtained are described in the publication of Chen et al. (Breast Cancer Res. Treat.) attached in the appendix section of this report. These techniques allow the analysis of normal and tumor template DNA from microscopic lesions. Our approach is based on the use of microsatellite chromosome markers. SSRs (simple sequence repeats) became tools of common use in the analysis of genetic abnormalities in carcinogenesis (Futreal et al., 1992; Jones and Nakamura, 1992). The frequent polymorphism in their length among different individuals makes SSRs particularly valuable for the detection of allelic losses or imbalance affecting specific chromosome areas. They also allow the identification of tumors that may be generated due to errors in DNA mismatch repair and characterized by a general microsatellite instability (Aaltonen, et al., 1993).

Interphase cytogenetics chromosomal *in situ* hybridization, or CISH is another recently developed technique being used extensively for the study of genomic abnormalities in solid tumors (Hopman, et al., 1991; Devilee, et al., 1988). This technique can also be applied to paraffin-embedded tissue sections (Emmerich, et al., 1989; Walt, et al., 1989; Dhingra, et al., 1992). CISH allows evaluation of the degree of intratumor clonal heterogeneity and eventually identification of tumor subpopulations on microscopic lesions (Dhingra, et al., 1992). The optimization of micromolecular techniques such as SSR analysis of chromosomal loci from paraffin-embedded sections, coupled with other techniques in current use such as interphase cytogenetics and conventional immunohistochemistry, will allow valuable retrospective studies of archival tissues to be done.

In the report of Chen et al. (Breast Cancer Res. Treat., appendix) we described a technical approach that allows the study of numerous chromosomal loci (n=20-50) from single paraffin-embedded tissue sections by microsatellite length polymorphism analysis. DNA samples from normal and breast cancerous tissue can be obtained from the same section by means of microdissection. This technique was further improved by subjecting DNA to several cycles of amplification with a degenerate (universal) primer and then with specific microsatellite primers. This amplified DNA was also used to screen for mutations in the p53 gene by means of PCR-SSCP. In addition adjacent tissue sections were used to asses specific chromosome copy number by interphase cytogenetic analyses (chromosome *in situ* hybridization) and to analyze expression of specific genes such as p53 and ERBB2. Details on the methodology employed can be found in the

appendix. As an example of the use of our approach we performed a detailed chromosome 17 allelotypic analysis in 22 breast tumors (5 ductal carcinomas *in situ*, 13 invasive ductal carcinomas, and 4 invasive lobular carcinomas). We detected mutations in the p53 gene by PCR-SSCP in 36% of the samples. Samples with significant levels of p53 protein accumulation detected by immunohistochemistry were also positive for mobility shifts in the SSCP analysis. We observed that chromosome 17 allelic losses and imbalance occurred at as early a stage as ductal carcinoma *in situ* (DCIS). Although in some cases we observed allelic losses or imbalance affecting the 17p13 region, close to the p53 locus, several of the tumors showed dissociation between such loss or imbalance and p53 mutation. Lobular carcinomas were predominantly disomic for chromosome 17 in contrast with ductal tumors, which often showed polysomy for chromosome 17. This comprehensive approach correlating the tumor subtype, its allelotype, with specific chromosome copy number and specific gene mutations and expression in preinvasive or early invasive breast cancer lesions will potentially provide information of relevance for a better understanding of the multistep mechanisms of breast carcinogenesis.

Comparative studies at preinvasive and invasive stages of breast cancer development and microsatellite instability as a marker of a breast cancer subtype.

As indicated in the introduction in spite of the abundance of data the relevance, role and timing of most of the genetic abnormalities observed in sporadic breast cancer are still unclear. It is also not known whether specific mutations play relevant roles as causative factors or are the consequence of the general genomic instability and progression in breast tumors. Most cytogenetic and molecular information on breast cancer has been obtained by analysis of advanced invasive carcinomas and metastases. In addition, very few studies have discriminated between the different histologic types of breast cancer. Therefore in a **second phase** of our project we focused our studies on relatively early stages of breast cancer progression by analyzing preinvasive lesions (ductal carcinoma *in situ* (DCIS), as well as comparing the allelotype of the two major histological subtypes of invasive breast carcinomas, i.e. ductal and lobular. The results of these studies resulted in another publication currently in press, (Aldaz, et al., Cancer Res. in press). A preprint of this manuscript can be found in the appendix section. Briefly, we compared the allelotypic profile of 23 *in situ* ductal carcinomas with that of 29 invasive ductal carcinomas. We also compared the allelotype of the invasive ductal breast carcinomas with that of 23 invasive lobular breast carcinomas. These studies were performed by means of microsatellite length polymorphisms from microdissected paraffin sections. We observed that involvement of chromosome arms 1p, 3p, 3q, 6p, 16p, 18p, 18q, 22q, and possibly 6q and 11p appear to be late events in breast cancer progression since allelic losses or imbalances affecting these areas were observed with very low frequency at the *in situ* stage (data summarized in Figure 1). On the other hand allelic imbalances and losses affecting chromosome arms 7p, 16q, 17p and 17q appear to be early abnormalities since they were observed in approximately 25-30% of DCIS lesions (see Figure 1 and Aldaz, et al., in appendix). Allelic losses and imbalances affecting the 8p arm were frequently observed in invasive lobular breast carcinomas. Interestingly, we also observed that microsatellite instability, also known as replication error phenotype (RER+), was found to occur at a high frequency in invasive lobular breast carcinomas, since 9 out of 23 (39%) were RER+, compared with 7 of 52 (13.5%) RER+ of breast cancers with ductal differentiation (p value = 0.012, chi-square test). Our findings provide for the first time molecular evidence suggesting that

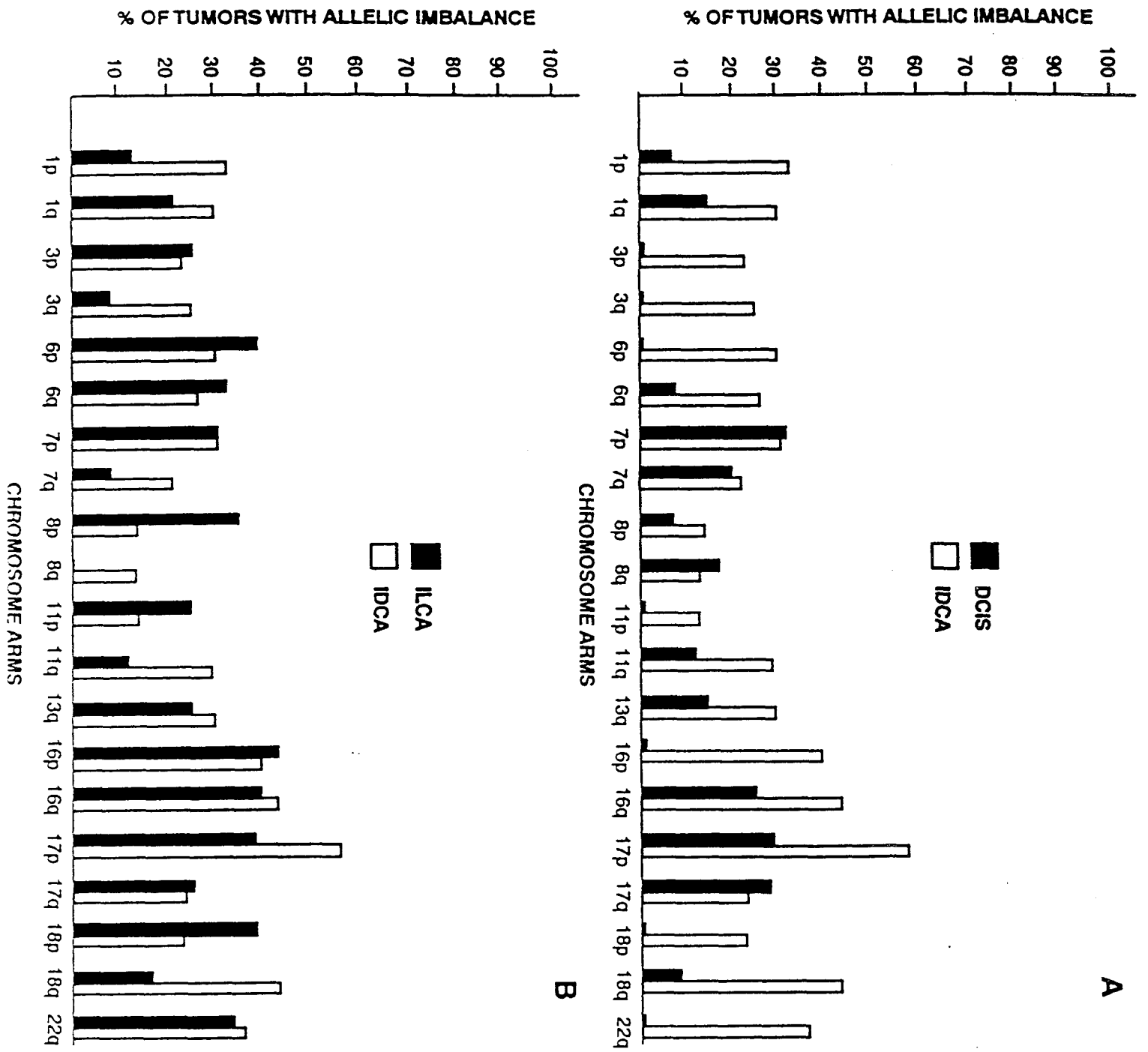

Figure 1. A). Comparative allelotype of breast ductal carcinomas *in situ* (DCIS), n= 23, versus invasive ductal carcinomas (IDCA), n= 29. B). Comparative allelotype of invasive breast ductal carcinomas (IDCA), n=29 with that of invasive lobular carcinomas (ILCA) n= 23.

invasive lobular breast carcinomas may arise by a different mechanism of carcinogenesis than ductal carcinomas. Further detailed description of the methodology use and these results is provided in the attached publication in the appendix section (Aldaz et al, Cancer Research, in press.).

Chromosome 9p and *p16/CDKN2* studies in breast cancer

In the introduction section and above we have indicated the chromosome arms most commonly affected in breast cancer by allelic losses. Loss of heterozygosity has been classically viewed as the indirect evidence for the possible existence of a tumor suppressor gene within a region affected by loss of alleles. One chromosome region that undergoes hemizygous and homozygous deletions in a variety of tumor types is 9p21-22 (van der Riet, et al., 1994; Aoki, et al., 1994; Merlo, et al., 1994; Knowles, et al., 1994). Detailed analysis of this region has shown that it contains an inhibitor of the cell cycle, the cyclin dependent kinase-4 inhibitor (*CDKN2*) gene, commonly referred to as p16 (Kamb, et al., 1994). Further analysis has revealed that this gene is frequently homozygously lost or deleted in cell lines derived from many tumor types, including astrocytoma (82%), bladder carcinoma (33%), lung carcinoma (25-32%), glioma (71-88%), melanoma (58-62%), renal carcinoma (56%) and breast carcinoma (60%) (Kamb, et al., 1994; Nobori, et al., 1994; Spruck, et al., 1994). This evidence implicated *CDKN2* as a putative tumor suppressor. However, since these studies were performed with cells grown *in vitro,* and since losses within 9p are infrequent in some tumor types, the relevance of *CDKN2* in these tumors was still questionable. To address this question, mutational analysis of *CDKN2* by sequencing has been conducted revealing alterations in uncultured tumors including esophageal carcinoma (52%), lung carcinoma (30%), and pancreatic carcinoma (38%), thus supporting the role of *CDKN2* as a tumor suppressor (Mori, et al., 1994; Hayashi, et al., 1994; Caldas, et al., 1994). Conversely, some tumor types have shown none or few mutations while lines derived from these types have shown high frequency of *CDKN2* deletions (Ohta, et al., 1994).

To ascertain whether chromosomal region 9p21-22 is also affected in breast cancer, and further determine if *CDKN2* plays a role in breast carcinogenesis, we performed allelotyping of the short arm of chromosome 9, single-strand conformational polymorphism (SSCP) analysis of *CDKN2* exon 1, and sequencing of *CDKN2* exon 2 in 21 uncultured primary breast carcinomas. Additionally, since it has been suggested that some *CDKN2* deletions and mutations are due to an immortalization or *in vitro* adaptation phenomenon, we analyzed 4 immortal breast epithelial lines derived from normal epithelium for loss or mutation of *CDKN2*.

Of 24 primary breast carcinomas analyzed, we observed a high frequency (58%) of loss of heterozygosity or allelic imbalance affecting subregion 9p21-22 (Brenner and Aldaz, see appendix). Mutational analysis of *CDKN2* (*p16*) was performed to determine whether this gene was the target of such alterations. Of 21 tumors analyzed, only 1 showed a mutation of probable consequence, suggesting that *CDKN2* appears not to be the target of LOH and indicating the possible existence of another tumor suppressor gene within this region. Additionally, since it has been suggested that some *CDKN2* deletions and mutations could be due to an *in vitro* phenomenon, four immortal breast cell lines derived from normal epithelium, MCF10F, MCF12F, 184A1, and 184B5, were examined for loss or mutation of *CDKN2*. Two lines (MCF10F and MCF12F) showed

homozygous deletions of *CDKN2*, and one (184A1) revealed a hemizygous deletion and a nonsense mutation in the remaining allele. This could imply an important role of *CDKN2* in the control of immortalization or *in vitro* adaptation, and is the first evidence of such in nontumor derived cell lines. Additionally, this is the first report of frequent LOH in the 9p21-22 chromosome subregion of uncultured primary breast tumors (Brenner and Aldaz, see appendix).

CONCLUSIONS

In Summary, in the first phase studies we have developed and applied a methodology for analyzing large numbers of chromosomal loci from single paraffin-embedded sections of small preinvasive and invasive breast cancer lesions. The basic technique involves tissue microdissection and microsatellite length polymorphism analysis. We have further improved this approach conducting a first round of DNA PCR amplification first with a degenerate universal primer for total genome amplification and then with specific microsatellite primers. We observed that the DNA so obtained preserved the proportionality of the different alleles as found in the original sample. We also determined that DNA obtained from the same lesions and amplified with the universal primer could be used to screen for specific gene mutations such as in p53. In addition, tissue sections adjacent to those used for the micromolecular analysis were successfully used to assess specific chromosome copy number by interphase cytogenetic analysis (CISH) and to analyze the expression of specific genes by immunohistochemistry.

This type of comprehensive approach using archival paraffin-embedded tissues is allowing correlation of genetic changes (at both the chromosomal and molecular levels) with their phenotypic consequences in the same preinvasive and invasive lesions. Such an approach is being used to dissect the specific events involved in the multistep process of breast carcinogenesis.

An example of the application of this techniques is that shown in the paper of Aldaz et al. (Cancer Res. in press). *In this report we identified for the first time the chromosome arms most frequently affected by losses and imbalances at preinvasive stages of breast carcinogenesis and those allelic losses involved in more advanced stages of progression.* These studies indicated that allelic imbalances and losses affecting chromosome arms 7p, 16q, 17p and 17q appear to be early abnormalities since they were observed in considerable number of DCIS lesions. We are currently more precisely defining by deletion mapping the region of chromosome 16q that is involved at the DCIS stage. We are also extending our studies to the analysis of other putative breast premalignant lesions such as LCIS.

Allelic losses and imbalances affecting the 8p arm were more frequently observed in invasive lobular breast carcinomas when compared with invasive ductal carcinomas.

Interestingly, in the course of the allelotyping studies we observed that numerous lobular tumor samples showed frequent abnormalities in the allele size migration in polyacrylamide gels when compared with the matched normal controls. Abnormalities in size of simple sequence nucleotide repeats is a phenomenon described as microsatellite instability (Aaltonen, et al., 1993). This phenomenon has been described as a characteristic of tumors from patients carrying the autosomal dominant

predisposition to tumors of the colon and endometrium, known as hereditary nonpolyposis colon cancer (Aaltonen, et al., 1993). These studies led to the identification of a group of human DNA mismatch repair genes as the cause of such general genomic instability phenomenon. Germline mutations in either the Escherichia *coli mutS* homolog *hMSH2* or the *mutL* homologs *hMlH1*, *hPMS1* and *hPMS2* have been found in different subsets of hereditary nonpolyposis colon cancer kindreds (Fishel et al., 1993; Bronner, et al., 1994). Microsatellite instability, also known as replication error phenotype, has also been reported to occur at various frequencies in various sporadic neoplasias other than colon cancer, such as cancers of the endometrium (Risinger, et al., 1993), stomach (Han, et al., 1993), esophagus (Meltzer, et al., 1994), bladder (Gonzalez-Zulueta, et al., 1993) and other tissues. Yee et al. (1994) reported microsatellite instability in 20% of breast cancers. Recently, Glebov et al. (Glebov, et al., 1994) observed that individuals with a family history of breast cancer and with *p53* mutations had a higher frequency of abnormalities of chromosome 17 loci.

In our study (Aldaz, et al., see appendix) of unselected breast cancer cases and mostly dinucleotide repeat markers, we observed the replication error-positive phenotype (RER+) in 16 of the 75 breast cancer samples (21%). This figure is similar to that reported by Yee et al. (Yee, et al., 1994). Interestingly however, when analyzed by histological subtype, only 13% (7 of 52 tumors) of ductal tumors (DCIS plus invasive ductal tumors) showed the RER^+ phenotype, in contrast with 39% (9 of 23) of infiltrating lobular breast carcinomas. This difference is statistically significant by chi-square analysis (p value = 0.012). Furthermore, if we exclusively compare invasive ductal carcinomas with invasive lobular carcinomas the difference is still significant (p value = 0.036). To address whether the observed microsatellite instability could be simply the consequence of a more aggressive tumor phenotype, we plotted the allelic loss index for the DCISs, and the invasive ductal and lobular tumors, identifying those samples that were RER+. We observed that the lobular breast carcinomas do not appear to represent a more advanced tumor stage because overall they had a similar level of allelic losses as the invasive ductal tumors. In addition, some tumors with very few losses (low indices) were already RER+, including three at the DCIS stage.

Our data suggest that invasive lobular breast carcinomas appear to arise by a mechanism of carcinogenesis different from that of ductal breast carcinomas and may constitute a possible different pathologic entity. These findings also support previous observations of different clinical behaviors of lobular breast tumors and ductal tumors (Tavassoli, 1992; Silverstein, et al., 1994; Harris, et al., 1984). The diagnosis of lobular breast carcinoma has been associated with a higher risk for development of multifocal or subsequent contralateral breast cancer (Tavassoli, 1992; Silverstein, et al., 1994). The possibility exists that some patients that develop lobular breast tumors could harbor or develop mutations in any of the DNA mismatch repairs genes in the mammary epithelium, thus producing a field defect and constituting a facilitating event for the development of secondary mutations leading to tumor development.

It is of importance to analyze the role of microsatellite instability in breast cancer in light of the findings of Glebov et al. (1994), who reported an association between microsatellite instability and familial history of breast cancer. The samples we used, however, were obtained at random from pathology files, and detailed information on familial history of breast cancer was not available for most of the cases, so we can not evaluate in detail at this

point the association between microsatellite instability and family history. Nevertheless, to at least partially address this point, we conducted telephone interviews with the patients (or their next of kin), who had breast tumors with the RER+ phenotype. We obtained detailed family histories on all first and second degree relatives (see Aldaz et al., appendix). Most of the breast cancers observed in family members however, were among older relatives, suggesting that these are probably sporadic breast cancers. Interestingly, four of the nine patients with lobular breast cancer and the RER+ phenotype had previously or synchronously, another breast cancer. In addition, three of these four cases were among those with multiple affected chromosomal loci. Based on our observations we speculate that detection of microsatellite instability has the potential to be useful in identifying patients at risk of developing second breast cancers. However, these are only observations and these findings will be certainly be substantiated with a larger data set. We currently are attempting to identify the source of such microsatellite instability phenomenon by performing a mutational analysis of specific DNA mismatch repair genes in RER+ tumors.

In summary our findings provide for the first time molecular evidence suggesting that invasive lobular breast carcinomas may arise by a different mechanism of carcinogenesis than ductal carcinomas and detection of microsatellite instability has potential to be used as a diagnostic-prognostic indicator.

In other studies (Brenner and Aldaz, see appendix) we have also identified the chromosome 9p21 region as a site frequently affected by allelic losses or imbalances in invasive breast cancer. This region showed to be affected in 58% of the tumors analyzed. However we did not find frequent mutations affecting the putative suppressor gene *p16/CDKN2* that maps to this region. This could indicate that a different suppressor gene of importance in breast cancer may reside in the 9p21 region. However, the possibility exists that the frequency of alterations of *p16/CDKN2* is actually higher since some mutations located outside of the coding region or hypermethylation of this gene could affect expression. Alternatively, detection of mutations might be masked by DNA from other cells in the heterogeneous tumor cell population or by infiltrating normal tissue, as suggested by Kamb et al. (1994). However, studies using methodology similar to ours in other uncultured tumor types have been conducted and have revealed a significant number of mutations (Mori, et al., 1994; Hayashi, et al., 1994; Caldas, et al., 1994). Therefore, we can assume that most mutations are not escaping detection. Alternatively, it may be that another tumor suppressor gene of significance in breast cancer could reside within this chromosomal subregion. This is supported by findings of similar discrepancies in primary tumors of other tissue types (Cairns, et al., 1994). Further studies are being conducted to evaluate other possible mechanisms for p16 inactivation during breast carcinogenesis and to better define the nature of the chromosomal abnormalities affecting the 9p21 region. Nevertheless we have strong evidence from in vitro studies suggesting that *p16* mutations could be a critical event for normal mammary epithelial cells to escape senescence. Studies are underway in our laboratory to specifically determine if loss of *CDKN2* is sufficient for immortalization and whether restoration of *CDKN2* expression in the aforementioned lines will induce senescence.

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Chromosome 9p Allelic Loss and *p16/CDKN2* in Breast Cancer and Evidence of *p16* Inactivation in Immortal Breast Epithelial Cells[1]

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ABSTRACT

To define the extent of involvement of chromosome 9p in breast carcinogenesis, we performed microsatellite length polymorphism analysis of markers spanning this region. Of 24 primary breast carcinomas analyzed, we observed a high frequency (58%) of loss of heterozygosity or allelic imbalance affecting subregion 9p21–22. Mutational analysis of *CDKN2* (*p16*) was performed to determine whether this gene was the target of such alterations. Of 21 tumors analyzed, only 1 showed a mutation of probable consequence, suggesting that *CDKN2* appears not to be the target of loss of heterozygosity and indicating the possible existence of another tumor suppressor gene within this region. Additionally, since it has been suggested that some *CDKN2* deletions and mutations could be due to an *in vitro* phenomenon, four immortal breast cell lines derived from normal epithelium, MCF10F, MCF12F, 184A1, and 184B5, were examined for loss or mutation of *CDKN2*. Two lines (MCF10F and MCF12F) showed homozygous deletions of *CDKN2*, and one (184A1) revealed a hemizygous deletion and a nonsense mutation in the remaining allele. This could imply an important role of *CDKN2* in the control of immortalization or *in vitro* adaptation and is the first evidence of such in nontumor-derived cell lines. Additionally, this is the first report of frequent loss of heterozygosity in the 9p21–22 chromosome subregion of uncultured primary breast tumors.

INTRODUCTION

Breast cancer is the most common malignancy in American women, affecting as many as one in eight, and responsible for as many as one in five cancer-related deaths of women (1, 2). It has, therefore, become essential to define the molecular events resulting in breast carcinogenesis. Various chromosomes have been observed to be affected by a higher frequency of structural or numerical abnormalities in breast cancer. At the molecular level, several somatic mutations have also been described affecting various oncogenes and tumor suppressor genes. Allelic losses at variable frequencies have been reported for numerous chromosome subregions, the most common being 1p34–36, 1q23–32, 3p21–25, 6q, 7q31, 11p15, 13q14, 16q, 17p13, 17q, and 18q (3–14). LOH[3] has been classically viewed as the indirect evidence for the possible existence of a tumor suppressor gene within a region affected by loss of alleles.

One chromosome region that undergoes hemizygous and homozygous deletions in a variety of tumor types is 9p21–22 (15–18). Detailed analysis of this region has shown that it contains an inhibitor of the cell cycle, the cyclin dependent kinase-4 inhibitor (*CDKN2*) gene, commonly referred to as *p16* (19). Further analysis has revealed that this gene is frequently homozygously lost or deleted in cell lines derived from many tumor types, including astrocytoma (82%), bladder carcinoma (33%), lung carcinoma (25–32%), glioma (71–88%), melanoma (58–62%), renal carcinoma (56%), and breast carcinoma (60%; Refs. 19–21). This evidence implicated *CDKN2* as a putative tumor suppressor. However, since these studies were performed with cells grown *in vitro* and since losses within 9p are infrequent in some tumor types, the relevance of *CDKN2* in these tumors was still questionable. To address this question, mutational analysis of *CDKN2* by sequencing has been conducted revealing alterations in uncultured tumors including esophageal carcinoma (52%), lung carcinoma (30%), and pancreatic carcinoma (38%), thus supporting the role of *CDKN2* as a tumor suppressor (22–24). Conversely, some tumor types have shown none or few mutations, while lines derived from these types have shown high frequency of *CDKN2* deletions (25).

To ascertain whether chromosomal region 9p21–22 is also affected in breast cancer and further determine if *CDKN2* plays a role in breast carcinogenesis, we performed allelotyping of the short arm of chromosome 9, SSCP analysis of *CDKN2* exon 1, and sequencing of *CDKN2* exon 2 in 21 uncultured primary breast carcinomas. Additionally, since it has been suggested that some *CDKN2* deletions and mutations are due to an immortalization or *in vitro* adaptation phenomenon, we analyzed four immortal breast epithelial lines derived from normal epithelium for loss or mutation of *CDKN2*.

MATERIALS AND METHODS

Tissue Samples, Cell Lines, and DNA Extraction. Normal and tumor breast samples were obtained from the Cooperative Human Tissue Network. Samples were snap frozen with liquid nitrogen less than 1 h after surgery. Cell lines MCF-10F (CRL10318), MCF-12F (CRL10783), 184A1 (CRL8798), and 184B5 (CRL8799) were purchased from American Type Culture Collection (Rockville, MD) and cultured as described elsewhere (26, 27). Total genomic DNA was isolated using phenol:chloroform:isoamyl (25:24:1) in Phase Lock Gel tubes (5 Prime→3 Prime, Boulder, CO), according to a standard protocol (28), and precipitated with 2.5 volumes of ethanol.

Microsatellite Length Polymorphism and *CDKN2* Deletion Analysis. PCR was performed using 60 μM individual end-labeled primer sets, 175 μM corresponding cold primer, 100–300 ng of template, 2 mM $MgCl_2$, 300 μM deoxynucleotide triphosphates, 1 × *Taq* buffer (Promega), 10% DMSO, and 1.5 units of *Taq* polymerase (Promega). Amplification was done using a hot-start protocol; DNA, primers, DMSO, and H_2O were heated to 95°C for 5 min and then brought to 80°C. Remaining ingredients were added, and cycling then proceeded with no extension step. Primers used were: *D9S199, D9S157, D9S171, D9S169, D9S165,* or *D9S15* (Research Genetics, Huntsville, AL). Products were resolved on 7% sequencing gel, and autoradiograms were developed after 12–48 h exposure. Partial LOH and allelic imbalance were considered significant only if the signal intensity of one allele was diminished by approximately one-half or more of its normal intensity in relation to the remaining allele; complete LOH was defined as a decrease of more than 90% in the signal intensity of one allele relative to the other.

Loss of *CDKN2* was determined by duplex PCR using unlabeled primers for exon 2 mentioned below for sequencing, simultaneously with control microsatellite marker *D13S155*. Amplification was done as described above for microsatellites. The resulting products were resolved by electrophoresis on a 2–3% NuSieve (FMC) agarose gel in 1X TAE.

Sequencing and PCR-SSCP. Exon 1 was analyzed by PCR-SSCP and sequenced as reported previously (29). Exon 2 of the *CDKN2* gene was amplified by PCR using the primers 5′-ACCATTCTGTTCTCTCTGGC-3′ and 5′-CTCAGATCATCAGTCCTCAC-3′. Amplification products were resolved on 2% NuSieve agarose (FMC), bands were excised, and DNA was recovered using a PCR Preps kit (Promega). Sequencing reactions were then

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[3] The abbreviations used are: LOH, loss of heterozygosity; SSCP, single-strand conformational polymorphism; CDK4, cyclin-dependent kinase-4.

performed using a fmol cycle sequencing kit (Promega) with the same primers and inside primers 5′-CACCAGCGTGTCCAGGAA-3′ and 5′-CGATGCCT-GGGGCCGTCT-3′. Because of the GC-rich nature of the sequence, excess deoxynucleotide triphosphates and 1 unit Terminal Transferase (USB) were added, and nonterminated products were extended at 37°C for 30 min. Products were resolved on a 5% sequencing gel and autoradiograph developed after 12–96 h exposure.

RESULTS AND DISCUSSION

Previous allelotypic studies have not implicated the short arm of chromosome 9 as a frequent target for allelic losses in breast cancer (3–14). But most of these earlier studies were based on RFLP analysis by means of Southern blotting with a very limited number of molecular markers for chromosome 9p (6, 14). Interestingly, more recent studies using microsatellite length polymorphism analysis have demonstrated 9p loss in a variety of tumor types. These include gliomas (30), melanomas (31), bladder carcinomas (32), lung carcinomas (17), and most recently, preinvasive and malignant head and neck squamous cell carcinomas (15). These losses have been localized to region 9p21–22 (33). To ascertain whether this same region is affected in breast cancer, we used five polymorphic microsatellite markers spanning the p arm and one marker on the q arm to assess LOH at chromosomal region 9p21–22. Marker *D9S199* is located in the telomeric region of 9p; 10 cM proximal in 9p21–22 is marker *D9S157*; another 7cM proximal in 9p21 is marker *D9S171*; 7 cM further is *D9S169*, and *D9S165* is located between 9p13 and marker *D9S15* at 9q13 (Fig. 1). Interestingly, of the 24 tumors we analyzed, allelic imbalances or LOH was observed in 14 tumors (58%; Fig. 1), and at least one-half of the tumors showed allelic imbalance or LOH of multiple markers (Figs. 1 and 2). Most losses observed affected markers *D9S169* and *D9S171*, which showed frequencies of LOH or imbalance of 58 and 53% of informative cases, respectively. Although a few cases clearly involve the entire short arm (A12, A19, and A23) or chromosome (A11), the majority of cases contain losses that begin or end on either side of these two markers. This indicates that the minimum area of overlap includes these two markers and that the target area lies somewhere in between. To our knowledge, there have been no previous reports of LOH or other alterations on chromosome 9p in uncultured primary breast tumors. However, our analysis indicates a high incidence of allelic abnormalities at chromosomal region 9p21 in breast cancer.

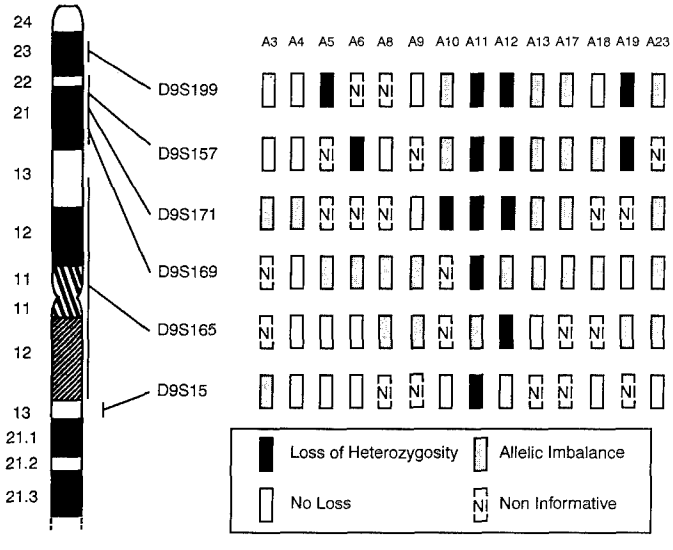

Fig. 1. Chromosome 9p allelic losses and imbalances in uncultured breast carcinomas.

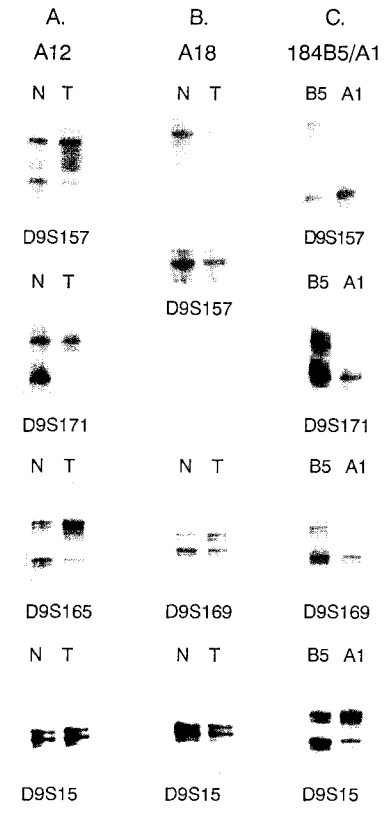

Fig. 2. Representative microsatellite analysis of two primary breast carcinomas (A12 and A18) and two chemically immortalized breast epithelial cell lines (184A1 and 184B5). *A*, tumor A12 shows loss of heterozygosity of three markers on chromosome arm 9p (*D9S157, D9S171,* and *D9S165*) while retaining heterozygosity at 9q13 (*D9S15*). *B*, tumor A18 shows an allelic imbalance of two 9p markers (*D9S157* and *D9S169*) and retains heterozygosity at 9q13 (*D9S15*). *C*, chemically immortalized cell line 184A1 shows complete loss of heterozygosity at three 9p markers as well as an allelic imbalance at 9q13 (*D9S15*).

On the basis of this relatively high rate of alterations at subregion 9p21 and on the basis of the previous report of Kamb *et al.* (19) indicating *CDKN2* as one obvious target of allelic loss in this region, we decided to search for alterations in the *CDKN2* gene. All tumors were examined, except tumors A22 and A24 (which incidentally showed no alteration of chromosome 9p) and tumor A11, to which sufficient DNA was not available. By direct sequencing of exon 2, we found only three alterations, of which only one was of probable consequence. This was a missense mutation observed in codon 75 (tumor A1; Fig. 3*A*), resulting in an amino acid change from histidine to tyrosine. This mutation had been reported previously (29) and is not suspected of being a polymorphism. Of additional interest is that this tumor (A1) showed no allelic loss on the p arm of chromosome 9 (Fig. 1). Of the two other mutations found, one was a missense change from alanine to threonine in codon 140 (tumor A9), a frequently reported probable polymorphism apparently not involved in cancer (34), and the second, a silent mutation in codon 65, resulting in no change of amino acid sequence (tumor A13). The remaining 18 tumors revealed no mutations by sequencing of exon 2. Although previous reports indicate that the majority (~90%) of mutations of *CDKN2* occur in exon 2 (19, 34), we performed PCR-SSCP analysis of exon 1 to be sure no mutations were present. To facilitate detection, two different conditions were used as described in a previous report (29). Nevertheless, we observed no mobility shifts in any of the 21 tumors analyzed (data not shown). To corroborate our PCR-SSCP results, we also performed sequencing of exon 1 in some tumors (A10, A12, A16, and A23) and found no base changes.

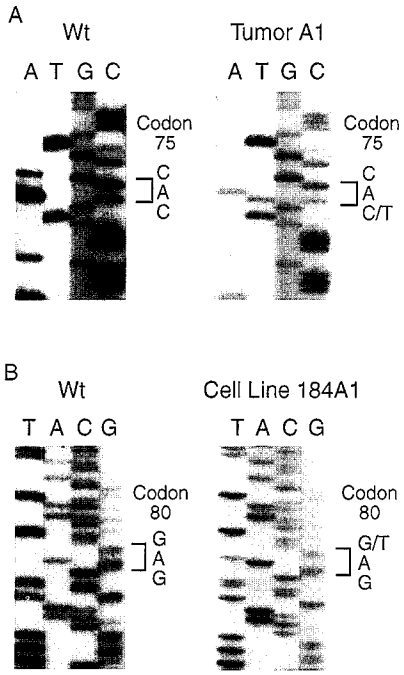

Fig. 3. Mutational analysis by sequencing of *CDKN2* exon 2. *A*, tumor A1 shows a missense mutation at codon 75, resulting in an amino acid change from histidine to tyrosine. *B*, immortal nontumorigenic breast epithelial cell line 184A1 shows a nonsense mutation at codon 80, resulting in a truncated transcript.

Despite the high rate of allelic imbalance affecting the 9p21 region, the aforementioned results indicate that *CDKN2* is not frequently mutated in these breast tumors. This low rate of mutation is in agreement with a previous report that used PCR-SSCP exclusively (35) and suggests that mutations of *CDKN2* are not a critical event in breast carcinogenesis and that this gene is probably not the target of allelic loss.

A discrepancy exists between the high rate of loss of *CDKN2* in cell lines derived from certain tumor types and the rarity of alterations in the corresponding primary tumors themselves. This is exemplified by the contradiction between the finding of Kamb *et al.* (19) of *CDKN2* loss in >60% of cell lines derived from breast carcinomas and our findings of few mutations or homozygous loss in primary breast carcinomas themselves. To help address this issue, we analyzed four nontumorigenic immortal cell lines. Two of these cell lines, MCF-10F and MCF-12F, are spontaneously immortalized cell lines produced by long-term culture of normal mammary epithelial tissue (26). The other two cell lines, 184A1 and 184B5, are independently derived from normal mammary epithelial cells from one patient and immortalized by treatment with benzo(*a*)pyrene (27). Of the four cell lines, both MCF-10F and MCF-12F showed complete loss of both *CDKN2* alleles. As can be observed in Fig. 4, both lines failed to amplify exon 2 of *CDKN2* in the presence of a control primer set in a duplex PCR reaction. Furthermore, in independent testing using radioactively labeled primers for PCR-SSCP analysis, both lines failed to amplify exon 1 of *CDKN2* (data not shown). Additionally, both cell lines were also analyzed for the presence of microsatellite markers spanning chromosome 9p. We observed that markers proximal and distal to *CDKN2* retained heterozygosity (data not shown). However, marker *D9S171* (heterozygosity score, 0.79) was found to be noninformative in both cell lines. Since other normal tissues from these patients were not available, we could not determine the constitutive genotype at this locus. Nevertheless, the homozygous loss of *CDKN2* in MCF10F and MCF12F appears to be the consequence of localized deletion events affecting both homologous chromosome arms. Interestingly, karyotypic analysis has shown (26) that cell line MCF-10F has a balanced reciprocal translocation, t(3;9)(3p13:9p22). However, it is not known whether this translocation "correlated in time with the acquisition of immortality," although it is known to have happened early in culture (26).

Further evidence suggesting that *CDKN2* may be a specific target for abnormalities while *in vitro* was provided by direct sequencing mutational analysis of the chemically [benzo(*a*)pyrene] immortalized line 184A1, which revealed a nonsense mutation at codon 80 (Fig. 3*B*). In addition, LOH at all three microsatellite markers in the 9p21 subregion was observed (Fig. 2*C*). These findings appear to indicate that one allele of *CDKN2* in 184A1 was deleted and that the remaining allele was inactivated through a nonsense mutation. Furthermore, it is possible that the mutation observed is the direct result of the benzo(*a*)pyrene treatment. We did not observe, however, any mutations of exons 1 or 2 of *CDKN2* in the 184B5 cell line by direct sequencing analysis. The possibility does exists that a mutation or deletion exists in another gene, such as the retinoblastoma gene, thereby circumventing the need for *CDKN2* inactivation. Experiments are in progress to address this point.

Previous work has shown that *CDKN2* alterations are at least three times more common in tumor-derived cell lines than in uncultured tumors. However, to our knowledge, there has been no analysis of *CDKN2* in cell lines derived from normal tissue. Here we show that *CDKN2* was affected in three of four cell lines (75%) derived from "normal tissue." This may help explain the aforementioned discrepancy between the high rate of loss of *CDKN2* in cell lines derived from certain tumor types and the rarity of alterations in the corresponding primary tumors themselves. It would be reasonable to assume that to escape senescence, cell lines would need to mutate or lose genes that play an important role in restricting cell cycle progression. *CDKN2* has been shown to be an important inhibitor of the cell cycle that acts to block progression through G_1 by inhibiting the kinase activity of CDK4. The kinase activity of CDK4 is able to phosphorylate important regulatory proteins, such as Rb, and prevents them from binding and inactivating their associated transcription factors, thereby stimulating growth. Should CDKN2 be deleted or inactivated by mutation, *CDK4* would be free to propel the cell through the cell cycle and into cell division. This would be analogous to the loss of p53 in immortalization (36), since p53 acts to regulate cell cycle progression through transcriptional activation of $p21^{waf-1}$, an inhibitor of CDK2. Thus, the implication is that although *CDKN2* may not play a significant role in breast carcinogenesis, it may be important in the control of immortalization or *in vitro* adaptation. This explanation is supported by recent findings of Loughran *et al.* (37) in

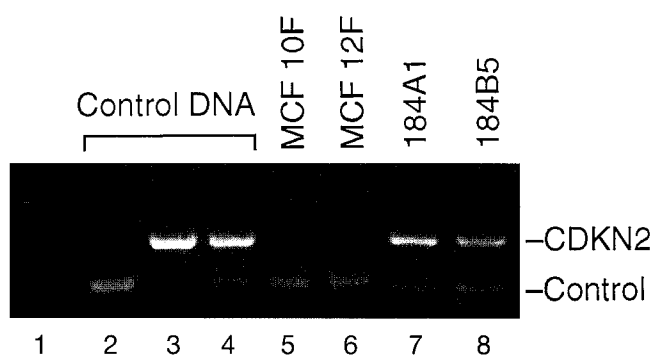

Fig. 4. Duplex PCR analysis of *CDKN2* showing homozygous deletion of exon 2 in nontumorigenic immortal breast epithelial lines MCF10F and MCF12F. *Lane 1*, molecular weight markers; *Lane 2*, control DNA with primer *D13S155*; *Lane 3*, control DNA with primer for *CDKN2*; *Lane 4*, control DNA with duplex PCR (*D13S155* and *CDKN2*); *Lanes 5–8*, duplex PCR on cell lines as indicated.

which loss of the 9p21 subregion was found to correlate with the acquisition of an immortal phenotype of neoplastic human head and neck keratinocyte cell lines. Most of the immortal lines analyzed in that report were derived from advanced tumors. Although loss of *CDKN2* does not constitute evidence that this is a senescence gene, our finding of loss in two nonneoplastic lines and loss with mutation of the remaining allele in another adds weight to this prospect.

In summary, in the present study, the frequency of alterations of the 9p21 region in uncultured breast tumors was high (≈60%), while the rate of mutation of *CDKN2*, a tumor suppressor gene located in this region, was significantly low to suggest that it is not the target of alteration in breast cancer. However, the possibility exists that the frequency of alterations of *p16/CDKN2* is actually higher since some mutations are located outside of the coding region, or hypermethylation of this gene could affect expression. Alternatively, detection of mutations might be masked by DNA from other cells in the heterogeneous tumor cell population or by infiltrating normal tissue, as suggested by Kamb *et al.* (38). However, studies using methodology similar to ours in other uncultured tumor types have been conducted and have revealed a significant number of mutations (22–24). Therefore, we can assume that most mutations are not escaping detection. Alternatively, it may be that another tumor suppressor gene of significance in breast cancer could reside within this chromosomal subregion. This is supported by findings of similar discrepancies in primary tumors of other tissue types (39). Further studies are needed to better define the extent of involvement of *CDKN2* in tumorigenesis. Studies are under way in our laboratory to specifically determine if loss of *CDKN2* is sufficient for immortalization and whether restoration of *CDKN2* expression in the aforementioned lines will induce senescence.

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Technical approach for the study of the genetic evolution of breast cancer from paraffin-embedded tissue sections

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Key words: breast cancer, allelotype, *in situ* hybridization, paraffin sections

Summary

We have optimized a technique that allows the study of numerous chromosomal loci (n = 20–50) from single paraffin-embedded tissue sections by microsatellite length polymorphism analysis. DNA samples from normal and breast cancerous tissue can be obtained from the same section by means of microdissection. This technique was further improved by subjecting DNA to several cycles of amplification with a degenerate (universal) primer and then with specific microsatellite primers. This amplified DNA was also used to screen for mutations in the p53 gene by means of PCR-SSCP. In addittion adjacent tissue sections were used to assess specific chromosome copy number by interphase cytogenetic analyses (chromosome *in situ* hybridization) and to analyze expression of specific genes such as p53 and ERBB2. As an example of the use of our approach we performed a detailed chromosome 17 allelotypic analysis in 22 breast tumors (5 ductal carcinomas *in situ*, 13 invasive ductal carcinomas, and 4 invasive lobular carcinomas). We detected mutations in the p53 gene by PCR-SSCP in 36% of the samples. Samples with significant levels of p53 protein accumulation detected by immunohistochemistry were also positive for mobility shifts in the SSCP analysis. We observed that chromosome 17 allelic losses and imbalance occurred at as early a stage as ductal carcinoma *in situ* (DCIS). Although in some cases we observed allelic losses or imbalance affecting the 17p13 region, close to the p53 locus, several of the tumors showed dissociation between such loss or imbalance and p53 mutation. Lobular carcinomas were predominantly disomic for chromosome 17 in contrast with ductal tumors, which often showed polysomy for chromosome 17. This comprehensive approach correlating the tumor subtype, its allelotype, with specific chromosome copy number and specific gene mutations and expression in preinvasive or early invasive breast cancer lesions will potentially provide information of relevance for a better understanding of the multistep mechanisms of breast carcinogenesis.

Introduction

Numerous somatic mutations affecting various genes and chromosome regions have been described in human breast cancer. However, the relevance and role in sporadic breast cancer of most of these abnormalities is still unclear [1–3]. It is very important to determine whether some of these anomalies are cause or effect of tumor progression [2]. Thus, there is a need for studies addressing the sequentiality and timing of the various genomic abnormalities from the putative breast premalignant lesions to the most aggressive malignant phenotypes. The best obvious source of material for the identification of the various stages of progression is

available from paraffin-embedded tissues used in routine diagnostic procedures.

In this report we describe the optimization of a comprehensive technical approach for a multiparametric analysis of human breast cancer lesions from paraffin-embedded tissue sections. By analyzing preinvasive and early invasive breast cancer lesions, this approach allows determination of the timing of presentation of several of the most common genomic abnormalities. The techniques described here allow the analysis of normal and pathological template DNA from microscopic lesions. Our approach is based on the use of microsatellite chromosome markers (simple sequence repeats or SSRs) for tissue allelotyping [4]. SSRs became tools of common use in the analysis of genetic abnormalities in carcinogenesis [5, 6]. The frequent polymorphism in their length among different individuals makes SSRs particularly valuable for the detection of allelic losses or imbalance affecting specific chromosome areas. They also allow the identification of tumors that may be generated due to errors in DNA mismatch repair and characterized by a general microsatellite instability [7].

Interphase cytogenetics chromosomal *in situ* hybridization, or CISH is another recently developed technique being used extensively for the study of genomic abnormalities in solid tumors [8, 9]. This technique can also be applied to paraffin-embedded tissue sections [10–12]. CISH allows evaluation of the degree of intratumor clonal heterogeneity and eventually identification of tumor subpopulations on microscopic lesions [12]. The optimization of micromolecular techniques such as SSR analysis of chromosomal loci from paraffin-embedded sections, coupled with other techniques in current use such as interphase cytogenetics and conventional immunohistochemistry, will allow valuable retrospective studies of archival tissues to be done.

Materials and methods

Microsatellite analysis

Five-to eight-micron-thick sections were cut from paraffin-embedded tissue blocks. Tissue microdissection was done on paraffin-embedded sections by drawing the silhouette of the area of interest on an H&E-stained slide and overlapping with the unstained specimen or by deparaffinizing first, staining with toloudine blue, and then microdissecting. Normal and tumor samples can be obtained from different areas of the same section or alternatively samples for normal DNA can be obtained from additional paraffin blocks from unaffected tissues (e.g. lymph nodes). After deparaffinizing (3X xylene/30 min) samples were rehydrated in decreasing alcohol gradients. DNA was extracted by incubating in 200 µl Instagene chelex matrix solution (BioRad) containing 60 µg of proteinase K. Incubations were carried out in a shaking incubator at 37° C overnight. After proteinase K digestion, samples were boiled for 10 min, vortexed, and centrifuged at > 7,000 G for 5 minutes. After centrifugation, 150 µl of usable volume was produced: of this, 2–10 µl were used for PCR amplification, depending upon the cellularity of each sample. Prior to PCR reactions, the forward primer was end labeled with T4 polynucleotide kinase (Promega) and 6,000 Ci/mmol [γ-^{32}P] = dATP (NEN). PCR reactions were performed in a 20 µl reaction volume 150 µM each dNTP, 1 u Taq polymerase, 1 X Taq buffer (Promega), $MgCl_2$, 1 pmol labeled primer and 2.5 pmol unlabeled forward and reverse primers.

In a 'hot start' procedure template and primers were heated to 96° C and denatured for 5 min. The remaining reaction constituents were added later at 80 ° C. The DNA was then subjected to 30–35 cycles of 40 sec at 94° C and 30 sec at 55° C, and a final elongation step of 5 min at 72° C. Products were electrophoresed on a 7% polyacrylamide sequencing gel at 90 w constant power for 2–2 hr. Gels were dried at 65–70° C for 1–2 hr and exposed to X-ray film from 4 hr to overnight. For certain primer sets, the amplification conditions were further optimized by titrating the $MgCl_2$ concentration in the reaction buffer. In some cases it may be necessary to use higher annealing temperatures (60° C or 65° C). Primers used were: D17S513 [13], D17S579 [14], MPO [15], D127S784, D17S849 [16], and D17S520 (J. Weber, unpublished data).

An alternative approach was used to analyze ex-

tremely small lesions or to generate additional template DNA. This approach was based on the use of a degenerate universal primer (DOP-PCR), as described by Telenius *et al.* [17]. To this end a 1–2 µl sample from the original 150 µl template containing solution was obtained. This sample was used as template for one round of PCR amplification with the universal degenerate primer 5'-CCGACTCGAGNNNNNNATCTGG-3' [17]. The reaction mixture contains a template DNA sample, 1.5 µM universal primer, 200 µM each dNTP, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 0.1 mg/ml gelatin, and 2.5 U Taq polymerase in a 50 µl reaction volume.

For this alternative approach, reaction mixture were subjected to one cycle of 4 min at 93° C; 8 cycles of 1 min at 94° C, 1 min at 30° C, and 3 min at 72° C; 28 cycles of 1 min at 94° C, 1 min at 56° C, and 3 min at 72° C, and a final extension at 72° C for 10 min. The resulting PCR product was then used as the template (1 µl) for a second PCR using either the specific microsatellite primers as described above or the specific p53 exon primers for PCR-SSCP analysis.

PCR-SSCP analysis

PCR-SSCP analysis of exons 5–8 of the p53 gene was performed using a commercially available human p53 amplimer panel (Clontech Lab. Inc.) [18]. Each PCR was done in a 20 µl volume containing 3 pmol of each primer at a 1:3 labeled/unlabeled ratio (both primers were previously end labeled with γ-^{32}P), 300 µM dNTPs, 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (weight/volume) gelatin, and 1.0 U Taq DNA polymerase. Samples were overlaid with 25 µl of mineral oil and then amplified in 35 cycles of 1 min at 94° C, 1 min and 40 sec at 66° C, and 1 min at 72° C for extension. The reaction mixture was then mixed 1:1 with a solution containing 20 mM EDTA, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol. Samples were heated at 95° C for 5 min, chilled on ice, and immediately loaded onto a 6% acrylamide/Tris-borate-EDTA gel containing 6% glycerol (volume/volume). Gels were run at 10 W (0.5 W/cm) for 3–4 hr at room temperature. Autoradiography was performed overnight at room temperature without intensifying screens. Genomic DNA from control samples containing known wild-type and mutant p53 alleles were processed in parallel in every assay.

Chromosomal in situ hybridization

The methodology for interphase cytogenetic analysis has been previously described [12]. Briefly, sections ~~from cell blocks and~~ from breast tumor tissue were dewaxed in xylene, dehydrated in graded alcohol, baked at 80° C for 1 h, and treated with 0.4% pepsin (Sigma, St. Louis, MO) in 0.2 N HCl for 30–55 min. Following denaturation at 94° C for 4 min, hybridization was carried out overnight in 60% formamide, 2X SSC containing 5% dextran sulphate, 1 mg/ml salmon sperm DNA, and 0.8–1.0 ng/µl probe. After hybridization, the slides were washed in 50% formamide in 2X SSC (pH 7.0) at room temperature and then washed in 0.1X SSC at 37° C. The hybridization signal was detected by the immunoperoxidase technique using the Vectastain ABC kit (Vector) and diaminobenzidine (DAB) as the chromogen substrate, as previously described [12]. Signals were quantitated as previously described [19]. The number of signal spots on a minimum of 100 nuclei in a given area was counted using previously described criteria [19]. A minimum of five randomly chosen areas were counted on each slide from each cell block. The CI was calculated by dividing the total number of signal spots by the number of nuclei counted. In brief, the chromosome index (CI) was calculated for defined histological regions by dividing the total number of signal spots by the total number of nuclei counted. For disomic cells, the CI for any given chromosome in our experience is 1.0. To account for minor technical differences in hybridization efficiency from one experiment to the next and from one region to another, a CI of ≥ 1.20 was considered to represent polysomy and a CI of ≤ 0.80 monosomy.

When permitted by the size of the lesion, tumors were also analyzed by routine DNA flow cytometry to determine DNA index (DI).

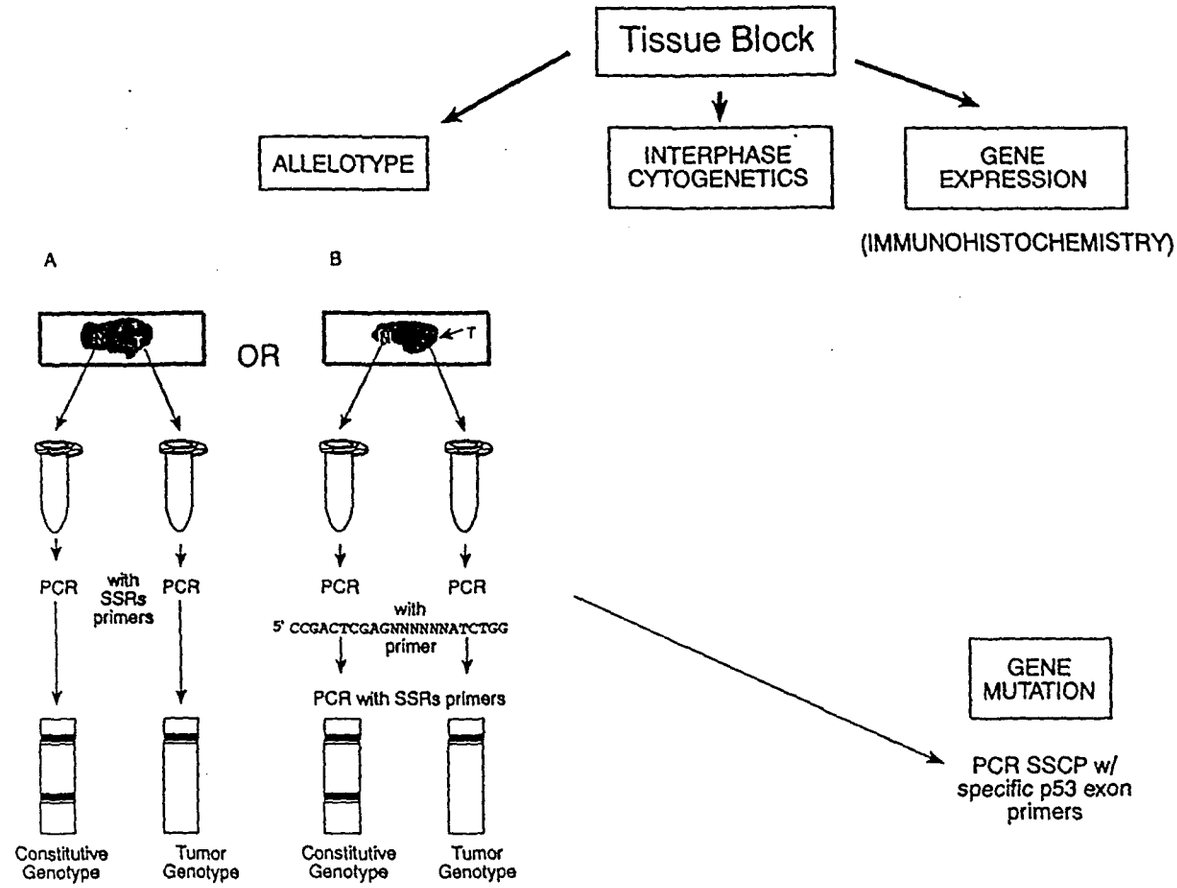

Fig. 1. Multiparameter analysis of breast cancer from paraffin sections to determine genotype phenotype correlations. A) DNA from normal (N) and tumor (T) areas of paraffin-embedded sections is obtained with the aid of microdissection. Normal DNA can also be obtained from a separate histological section of normal tissues from the same patient, e.g. unaffected lymph node. After microdissection of the area of interest the material is collected, purified and digested. PCR is performed using primers flanking specific microsatellite markers (SSRs). B) at right is shown the alternative protocol for extremely small samples using a few cycles of amplification with a universal primer mixture [17]. This DNA can also be used to screen for mutations such as in p53. Adjacent sections are used for interphase cytogenetics and immunohistochemistry.

Immunohistochemistry

p53 protein accumulation was analyzed by the avidin-biotin-peroxidase complex method using the D01 antibody (Oncogene Science), which detects both mutant and wild-type p53 protein. Results were expressed as the approximate percentage of positive cells in random microscopic fields of observation. ERBB2 expression was detected with a commercially available antibody (Oncogene Science).

Results and discussion

We have developed a technique to analyze multiple chromosomal loci from single, microdissected, paraffin-embedded sections. This involves PCR-mediated analysis of microsatellite length polymorphisms. Similar approaches were also recently developed by other laboratories [20]. We are now using the techniques described here to allelotype small preinvasive and invasive breast cancer lesions and then correlate the allelotypes with other tumor markers as well as cytogenetic changes such as nu-

merical abnormalities of specific chromosomes (Fig. 1). To overcome the potential problem of normal DNA contribution from stromal or inflammatory cells, we microdissected areas of interest from each tissue section. From 5–8 μm tissue sections, we obtained enough template DNA to perform approximately 20–50 PCR reactions, which allowed the analysis of as many different chromosomal loci (Fig. 1A). The number of reactions depends on the size of the original sample, and certain primer sets require higher levels of template DNA. Thus, this general strategy was further improved by modifying a previously described DOP-PCR technique [17] to allow the analysis of even smaller samples and the gathering of larger amounts of template DNA. This facilitated the analysis of more chromosomal loci as well as the screening of specific gene mutations by PCR-SSCP. We obtained a minimum sample (1–2 μl) from the original template-DNA-containing solution by this alternative approach (Fig. 1B). This sample was subjected to a few cycles of PCR amplification with a universal degenerate primer, as described by Telenius *et al.* [17]. The resulting amplified products were used as template for amplification with the specific microsatellite flanking primers or the gene-specific primers (e.g. p53 amplimer panel). Figure 2 compares both of the methods described above. The allelic loss affecting marker D17S579 (upper allele) could be detected equally well by the direct technique (Fig. 2A) and the universal primer technique (Fig. 2B). This indicates that the proportionality of the alleles is preserved even after several cycles of whole genome amplification with a universal primer. This was further demonstrated by mixing at variable proportions two DNA samples, each homozygous for a different allele of marker D17S513, and comparing the sensitivity of both techniques, the direct approach and the universal primer method (Figs 2C and D, respectively). The two samples with similar DNA concentrations were obtained from tissue sections were mixed at various proportions as indicated (Figs 2C, D) and were subjected to amplification using the direct approach (Fig. 2C). A 1 μl sample of the original mixtures was diluted in a 49 μl reaction volume for PCR amplification using the universal primer. Finally a 1 μl sample of this last reaction was

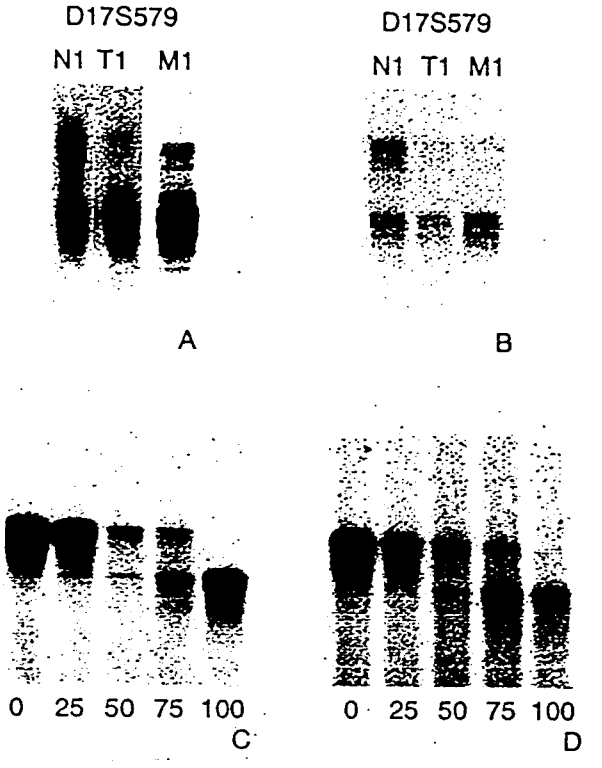

Fig. 2. Comparison of both techniques as shown in Fig. 1. A) Direct analysis of the D17S579 marker in patient 1, with loss of the upper allele in the tumor (T1) and in the corresponding metastasis (M1). At right B) is shown the analysis of the same D17S579 marker by the universal primer approach (Fig. 1B). As seen, the loss of the upper allele of this marker in T1 and M1 is preserved and detected using the universal primer approach.
Comparative analysis of mixtures of two alleles of D17S513 by using the direct method in C and the universal primer approach in D. Two DNA samples homozygous for D17S513 were mixed a various proportions prior to PCR, the percentage in the mixture of the sample with the smaller allele is shown at the bottom of each figure, see text for details.

used to amplify using the specific primer set for D17S513 (Fig. 2D). As can be observed both approaches yielded comparable results confirming that the universal primer method can detect alterations in the proportionality of the different alleles.

Figure 3 shows a representative analysis of multiple loci from the q and p arms of human chromosome 17 in various invasive and *in situ* breast carcinomas by the direct technique. Figure 4 shows the results of our detailed chromosome 17 analysis of 22 breast tumors (5 ductal carcinomas *in situ*, 13 invasive ductal carcinomas, and 4 invasive lobular carcinomas).

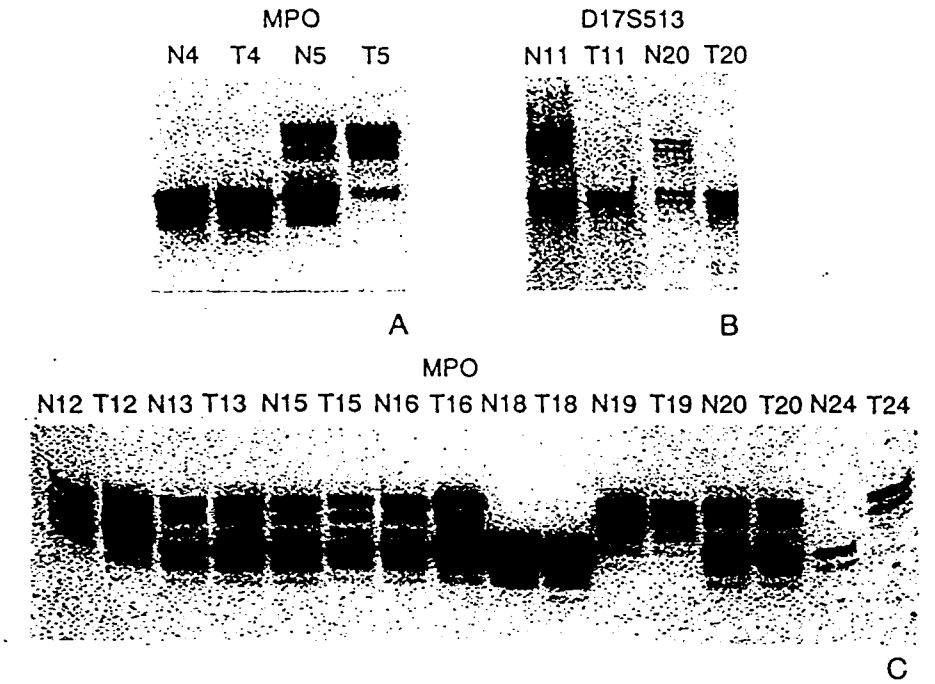

Fig. 3. Representative autoradiographs of multiple normal (N) and breast tumor (T) samples obtained from paraffin-embedded tissues demonstrating the analysis of chromosome 17 loci. In panels A and B note LOH in samples T5, T11, and T20. In panel C, note the generation of a novel allele (arrow) in the tumor T24; we observed the same phenomenon with other markers in this patient (microsatellite instability). PCR-amplified microsatellites with one primer end labeled were separated on a 7% polyacrylamide sequencing gel.

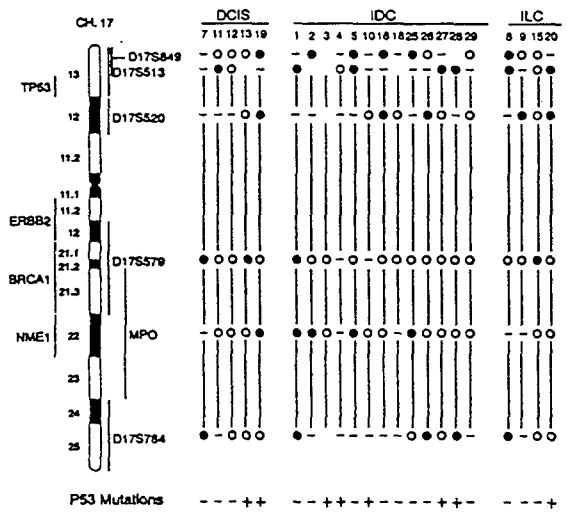

Fig. 4. Schematic representative summary of chromosome 17 allelotype and p53 mutation analysis of various breast cancer samples previously analyzed by means of microsatellite polymorphism in paraffin sections. Open circles, no LOH; closed circles, LOH or allelic imbalance; –, noninformative. p53 mutations were detected by PCR-SSCP analysis.

We also evaluated the sensitivity of the universal primer technique in detecting specific gene mutations. In this case we analyzed the same 22 tumors for p53 gene mutations by PCR-SSCP assay. We used 5 ng samples of template DNA from normal human DNA as negative controls and 5 ng samples from cell lines with known mutations in each of the p53 exons as positive control (e.g. Colo-320 for exon 7, BT-474 for exon 8). The control and tumor samples were first subjected to universal primer amplification as described in Methods. A 1–2 µl sample of the resulting products was then subjected to a second PCR using the end-labeled p53 exon-specific primers. After a final denaturation, the PCR products were separated on a 6% polyacrylamide gel under nondenaturing conditions. Representative results are shown in Fig. 5. Of the 22 tumors analyzed for mutations in p53 exons 5–8, we detected mobility shifts in 8 (36%) samples: 2 ductal carcinomas *in situ*, 4 invasive ductal carcinomas, and 1 lobular carcinoma (Fig. 4 lower panel).

Adjacent sections from 14 of the tissue blocks

were also analyzed for chromosome 17 copy number by interphase cytogenetics CISH and expression of p53 and ERBB2 by immunohistochemistry. The results of these studies are shown in Table 1. Based on the data shown in Fig. 4 and Table 1, we concluded that allelic losses and imbalance already were occurring at the carcinoma *in situ* stage, as previously reported [21]. In several cases we observed allelic losses or imbalance affecting the 17p13 region, close to the p53 locus, but in several other tumors this event and p53 mutations were dissociated as observed by other authors [22]. However, tumors showing significant levels of p53 protein accumulation such as tumors 7, 10, and 19 also showed mobility shifts in the p53 PCR-SSCP assay. We also observed only a modest number of losses involving the 17q21-22 region, lower than previously reported by others [5].

The results also show that interphase cytogenetic analysis supplements the information gained from ploidy analysis by DNA flow cytometry alone. In many cases where flow cytometry could not be performed because the lesions were too small for gross observation, CISH techniques allowed the copy numbers of specific chromosomes to be estimated (e.g. tumors 5, 10, 16, 19, and 24). Furthermore, difference in copy number of chromosomes between adjacent but phenotypically distinct regions could be determined: e.g. tumor 10, the *in situ* component was polysomic (CI 1.66) for chromosome 17, but the invasive component was disomic (CI 1.10).

In summary, we have developed and applied a methodology for analyzing large numbers of chromosomal loci from single paraffin-embedded sections of small preinvasive and invasive breast cancer lesions. The basic technique involves tissue microdissection and microsatellite length polymorphism analysis. We have further improved this approach conducting a first round of DNA PCR amplification first with a degenerate universal primer for total genome amplification and then with specific microsatellite primers. We observed that the DNA so obtained preserved the proportionality of the different alleles as found in the original sample. We also determined that DNA obtained from the same lesions and amplified with the universal primer could be used to screen for specific gene mutations such as in p53. In addition, tissue sections adjacent to those used for the micromolecular analysis were successfully used to assess specific chromosome copy number by interphase cytogenetic analysis (CISH) and to analyze the expression of specific genes by immunohistochemistry.

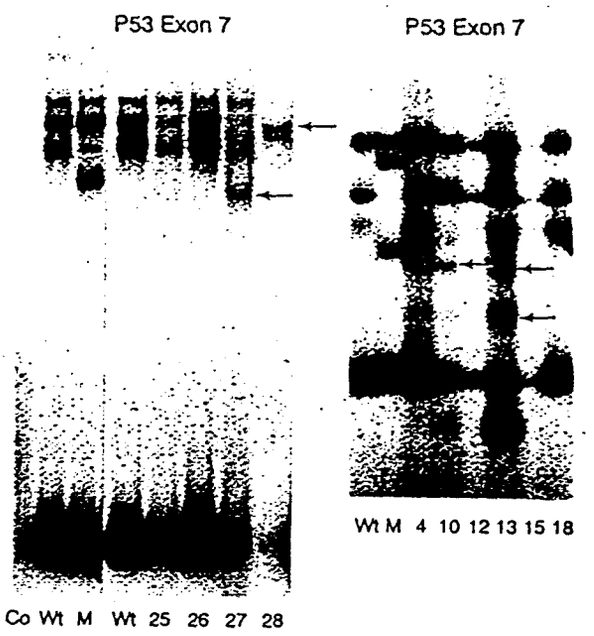

Fig. 5. Representative PCR-SSCP analysis of p53 exons 6 and 7 from a DNA template obtained from paraffin-embedded breast cancer tissues using the universal primer method. WT; wild type, M; positive control DNA for mutation in the corresponding exon. Note the clear shift in band mobility in some of the positive samples as indicated by arrows.

This type of comprehensive approach using archival paraffin-embedded tissues will allow correlation of genetic changes (at both the chromosomal and molecular levels) with their phenotypic consequences in the same preinvasive and invasive lesions. Such an approach will also allow us to dissect the specific events involved in the multistep process of breast carcinogenesis.

Acknowledgements

Supported by U.S. Army Award DAMD 17-94J-4078 and NIH grant P20 CA58186. We thank John Riley and Judy Ing for artwork and Michelle Gardiner for secretarial assistance.

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Table I. Analysis of multiple genotype and phenotype characteristics from breast cancer paraffin sections

Sample	Pathology[a]	Nuclear grade[c]	LOH or imbalance[d]		p53 mutation[e]	p53 accumulation[f]	c-erb B-2 expression[g]	DNA index[h]	Chromosome 17 CI[i]
			17p	17q					
4	IDC	I	○→	ni	+ (E6)	nd	nd	1.99	0.86
5	IDC	I	(2) ●	(1) ●	–	–	–	nd	0.91
10	IDC/DCIS	II	○→	○→	+ (E7)	5%	–	nd	IDC 1.10; DCIS 1.66
16	IDC	I	(2) ●	○→	–	5%	–	nd	1.60
7	DCIS[b]	II	ni	(2) ●	–	–	–	1.0	1.09
11	DCIS	I	(1) ●	○→	–	–	+	2.22	2.24
12	DCIS	I	○→	○→	–	–	+	1.36	nd
13 ~~1.19~~	DCIS	I	○→	(1) ← ●	+ (E7)	50%	+	1.13	← 1.14
19 ~~1.93~~	DCIS	II	(1) ← ●	○	+ (E8)	20%	–	nd	← 1.93
24	DCIS	II	MI[j]	MI[j]	nd	–	–	nd	nd
8	ILC	III	(2) ●	(1) ●	–	1%	–	1.16	1.12
9	ILC	II	(1) ●	○→	–	1%	–	1.0	1.05
15 ~~1.02~~	ILC	II	○→	(1) ← ●	–	–	–	0.93	← 1.02
20	ILC/LCIS	II	(2) ●	○→	+ (E8)	nd	–	1.65	1.21

[a] Abbreviations: IDC, invasive ductal carcinoma; DCIS, ductal carcinoma *in situ*; ILC, invasive lobular carcinoma; LCIS, lobular carcinoma *in situ*.

[b] All DCIS lesions were comedo DCIS with the exception of Sample # 7 which is a non-comedo lesion.

[c] According to Black's nuclear grading system in which I = poorly differentiated, II = moderately differentiated and III = well differentiated.

[d] According to microsatellite analysis, ●; loss or allelic imbalance, in parenthesis number of markers affected per arm; ○, no loss; ni, non informative.

[e] Exon affected, as determined by PCR-SSCP; shown in parentheses.

[f] Determined by immunohistochemistry using an antibody that recognizes both wild-type and mutant p53 protein. –, negative for accumulation; +, accumulation expressed as approximate percentage of positive cells; nd, not determined.

[g] Determined by immunohistochemistry.

[h] Determined by DNA flow cytometry from fresh specimens.

[i] Determined by chromosomal *in situ* hybridization with chromosome 17 centromeric probe.

[j] MI, microsatellite instability.

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Appendix

C. Marcelo Aldaz, M.D.
DAMD17-94-J-4078

[CANCER RESEARCH 55, ••••–••••, September 15, 1995]

Advances in Brief

Comparative Allelotype of *in Situ* and Invasive Human Breast Cancer: High Frequency of Microsatellite Instability in Lobular Breast Carcinomas[1]

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Abstract

To better understand the timing for presentation of allelic losses in human breast carcinogenesis, we compared the allelotypic profile of 23 *in situ* ductal carcinomas with that of 29 invasive ductal carcinomas. We also compared the allelotype of the invasive ductal breast carcinomas with that of 23 invasive lobular breast carcinomas. These studies were performed by means of microsatellite length polymorphisms from microdissected paraffin sections. We observed that involvement of chromosome arms 1p, 3p, 3q, 6p, 16p, 18p, 18q, 22q, and possibly 6q and 11p appear to be late events in breast cancer progression because allelic losses or imbalances affecting these areas were observed with very low frequency at the *in situ* stage. On the other hand, allelic imbalances and losses affecting chromosome arms 7p, 16q, 17p, and 17q appear to be early abnormalities because they were observed in approximately 25–30% of ductal carcinoma *in situ* lesions. Allelic losses and imbalances affecting the 8p arm were frequently observed in invasive lobular breast carcinomas. It was also interesting that microsatellite instability, also known as replication error (RER) phenotype, was found to occur at a high frequency in invasive lobular breast carcinomas because 9 of 23 (39%) were RER+, compared with 7 of 52 (13.5%) RER+ of breast cancers with ductal differentiation ($P = 0.012$, χ^2 test). Our findings provide for the first time molecular evidence suggesting that invasive lobular breast carcinomas may arise by a different mechanism of carcinogenesis than ductal carcinomas.

Introduction

Numerous studies have focused on the role of chromosome abnormalities and gene mutations in sporadic breast cancer, but to date no clear model of the critical events or delineation of primary abnormalities has emerged. Various chromosomes or chromosome subregions have been observed to be affected by a high frequency of structural or numerical abnormalities (1). At the molecular level, several somatic mutations have also been described (reviewed in Ref. 2). Amplification or overexpression of several oncogenes, growth factors, and cyclins has been observed at various frequencies (2). Specific allelic losses were also reported at various frequencies, at various chromosome regions, including 1p34–35, 1q23–32, 3p21–25, 6q, 7q31, 11p15, 11q22–23, 13q14, 16q, 17p13, 17q, 18q23–ter and 22q (3–12). Several of these areas appear to be the sites of putative tumor suppressor genes. The tumor suppressor gene *p53* is known to be mutated in a high percentage of breast cancers (13). Despite this abundance of data, the relevance, role, and timing of most of the described genetic abnormalities in sporadic breast cancer are still unclear. It is also not known whether specific mutations play relevant roles as causative factors or are the consequence of the general genomic instability and progression in breast tumors.

Most of the cytogenetic and molecular information on breast cancer has been obtained by analysis of advanced invasive carcinomas and metastases. In addition, very few studies have discriminated between the different histological types of breast cancer. We therefore focused this study on relatively early stages of breast cancer progression by analyzing preinvasive lesions (DCIS[3]), as well as comparing the allelotype of the two major histological subtypes of invasive carcinomas (*i.e.*, ductal and lobular).

These studies were performed by means of microsatellite length polymorphism analysis of paraffin-embedded tissue sections with simple sequence repeat markers for the chromosome subregions most commonly affected in breast cancer.

Materials and Methods

Paraffin blocks of breast tumors were randomly chosen from the archives of the Department of Pathology of The University of Texas M. D. Anderson Cancer Center. Five- to 8-μm-thick sections were cut from the blocks. The basic technical approach has been described previously (14). Briefly, normal and tumor samples were obtained from different areas of the same section by means of microdissection. After deparaffinization (three washes with xylene for 30 min each), the samples were rehydrated in decreasing concentration of alcohol. DNA was extracted by incubating each sample in 200 μl of Instagene chelex matrix solution (Bio-Rad, Hercules, CA) containing 60 μg of proteinase K in a shaking incubator at 37°C overnight. Then, the samples were boiled for 10 min, vortexed, and centrifuged at about 7000 × *g* for 5 min. Centrifugation produced 150 μl of supernatant, of which 2–10 μl was used for PCR amplification, depending on the number of cells in the sample. Before PCR, the forward primer was end labeled with T4 polynucleotide kinase (Promega Biotech, Madison, WI) and 6000 Ci/mmol [γ-^{32}P]dCTP (DuPont New England Nuclear, Boston, MA). PCR was performed in a 20-μl reaction volume including 150 μM each dNTP, 1 unit of Taq polymerase and 1× Taq buffer (Promega), $MgCl^2$, 1 pmol of labeled primer, and 2.5 pmol of unlabeled forward and reverse primers. A "hot-start" procedure was used in which the template and primers were heated in an initial denaturation step of 5 min at 96°C, and cooled to 80°C when the remaining reaction constituents were added, followed by 30–35 cycles at 94°C for 40 s, 55°C for 30 s, and a final elongation step of 72°C for 5 min. The products were electrophoresed on 7% polyacrylamide sequencing gels at 90 W constant power for 2–3 h. The gels were dried at 65–70°C for 1–2 h and exposed to X-ray film for 4 h to overnight. For certain primer sets, the amplification conditions were further optimized by adjusting the $MgCl^2$ concentration in the reaction buffer. The primers used (Research Genetics, Huntsville, AL) are described in Table 1.

The sample was considered to have partial loss of heterozygosity, or allelic imbalance, if the signal intensity of one allele was diminished by approximately one-half or more of its normal intensity (*i.e.*, in normal tissue) in relation to the remaining allele. Complete loss of heterogyosity was defined as a decrease of 90% or more in the signal intensity of one allele relative to the other.

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[3] The abbreviations used are: DCIS, ductal carcinoma *in situ*; RER+, replication error positive.

Table 1 *Frequency of allelic losses or imbalances in breast tumors by histology*

Marker	Location	No. of tumors affected/no. of informative loci (%) DCIS	IDCA	*P* value[a]	ILCA
D1S228	1p36–34	1/15 (7)	6/19 (32)	0.07	2/16 (12)
D1S213	1q31–32	3/20 (15)	7/23 (30)	NS	4/18 (22)
D3S1298	3p24.2–22	0/19 (0)	5/23 (22)	0.03	4/17 (24)
D3S1309	3q21.3–25.2	0/11 (0)	4/16 (25)	0.07	1/13 (8)
D6S276	6p22.3–21.3	0/11 (0)	3/10 (30)	0.05	4/10 (40)
D6S255	6q25.2	1/12 (8)	5/19 (26)	NS	4/13 (31)
D7S481	7pter–p15	4/12 (33)	5/16 (31)	NS	5/16 (31)
D7S550	7q36–qter	3/15 (20)	3/14 (21)	NS	1/13 (8)
D8S264	8p21–pter	1/15 (7)	2/14 (14)	NS	5/14 (36)
D8S256	8q24.13–qter	2/12 (17)	1 /8 (13)	NS	0 /9 (0)
HBB	11p15.4	0/12 (0)	2/14 (14)	NS	3/12 (25)
INT2(FGF3)	11q13.3	2/17 (12)	7/24 (29)	NS	2/16 (13)
D13S155	13q14.3–21.2	2/13 (15)	3/10 (30)	NS	2 /8 (25)
D16S407	16p13.13	0/15 (0)	8/20 (40)	0.005	7/16 (44)
D16S413	16q24.3	5/20 (25)	9/21 (43)	NS	6/15 (40)
D17S513	17p13	4/14 (29)	8/14 (57)	NS	6/16 (38)
D17S579	17q12–21.3	6/21 (29)	6/26 (23)	NS	9/16 (25)
D18S59	18pter–p11.22	0/14 (0)	3/13 (23)	0.06	6/15 (40)
D18S51	18q21.33	1/13 (8)	10/23 (44)	0.025	2/12 (17)
D22S283	22q12–13	0/14 (0)	5/14 (36)	0.01	6/17 (35)

[a] χ^2 test, 1 *df*. IDCA *versus* DCIS. NS, not significant.

Results and Discussion

DCIS and Invasive Ductal Carcinoma Allelotypes. One goal of this study was to determine which of the chromosome areas most commonly affected by allelic losses or imbalances in breast cancer were involved in the preinvasive stages of breast carcinogenesis. To that end we focused on the chromosome subregions reported to be affected in previous studies (2–12). We selected a panel of representative microsatellite markers mapping to those specific areas (Table 1). It is important to note that allelic losses and in particular allelic imbalances at specific loci do not necessarily imply the presence of a tumor suppressor gene in that area. Duplication of specific chromosome arms can also lead to an allelic imbalance. We view microsatellite length polymorphism analysis as a tool for measuring the general level of genomic instability at specific stages of tumor progression and also for identifying the chromosome arms affected at specific stages of progression. We analyzed with this approach a total of 75 breast cancer samples (23 DCIS, 29 invasive ductal carcinomas, and 23 invasive lobular carcinomas) at 20 different loci. The results obtained from the analysis of invasive ductal carcinomas validates the general approach because we observed similar frequencies of allelic losses to those reported previously (Refs. 2–12; Table 1; Fig. 1). We compared the incidence of allelic losses and imbalances in the DCISs and invasive ductal carcinomas to determine which chromosomal areas are already altered at the carcinoma *in situ* stage and which abnormalities are later events in ductal breast carcinogenesis. Seventeen of the 23 DCIS lesions (74%) had loss or imbalance of at least one locus (*i.e.*, only six of the tumors did not show any abnormality). The results are summarized in Fig. 1 and Table 1. No allelic losses were observed in any DCIS tumor affecting markers from chromosome arms 3p, 3q, 6p, 11p, 16p, 18p, 22q, and low frequency (5–15% of informative cases) for markers from arms 1p, 1q, 6q, 8p, 8q, 11q, 13q, and 18q. From Fig. 1 we can conclude that alterations in chromosome arms 1p, 3p, 3q, 6p, 16p, 18p, 18q, 22q, and possibly 6q and 11p appear to be late events in breast cancer progression because allelic losses or imbalances affecting these areas were not frequently observed at the DCIS stage. We can also conclude that allelic losses or imbalances affecting chromosome arms 16q, 17p, and 17q appear to be early abnormalities because they were observed in approximately 25–30% of DCIS. It was interesting that we also observed a considerable incidence of allelic imbalance affecting marker *D7S481*, which is on the short arm of chromosome 7, both in DCIS and invasive breast carcinomas (Fig. 1 and Table 1). This chromosome area was not reported previously to be frequently deleted in breast cancer. However, as mentioned previously, some of the imbalances observed could be the consequence of overrepresentation of chromosome 7p. Representative allelic losses and imbalances affecting the 16q marker *D16S413* are shown in Fig. 2*A*. Loss of alleles on 16q in invasive breast cancer has been reported by several groups (12, 15–17). At least two different regions on 16q have been reported to be involved in allelic loss and to possibly contain tumor suppressor genes (16, 17). Our findings also agree with those of previous cytogenetic studies that also implicated 16q as a possible site for primary chromosomal rearrangements in breast cancer (18, 19).

To our knowledge, this is the first report of a thorough allelotypic analysis of DCIS lesions. Previous reports have focused on the analysis of allelic losses on specific chromosome arms such as 17p (20) and more recently 11q (21). In agreement with the first of those reports (20), we observed 17p losses at the DCIS stage (Ref. 14, Fig. 1). On the other hand, we did not find a high incidence of losses at the DCIS stage (Fig. 1) affecting the *INT2* locus on 11q, as reported previously (21). This discrepancy probably is due to the fact that the study of Zhuang *et al.* (21) was performed on microdissected carcinoma *in situ* component of invasive tumors, whereas our study was performed on pure DCIS tumors with no invasive components.

Because DCIS lesions are a heterogeneous group in which the architectural pattern, nuclear grade, and presence of necrosis are thought to be prognostically important (22), the lesions we studied were subclassified according to the presence or absence of necrosis and nuclear grade. They were classified by a nuclear grading system into two groups: high-grade and non-high-grade DCIS. High-grade applied to poorly differentiated tumors and non-high-grade to moderate-to-well differentiated lesions. We also established an index of allelic loss or imbalance for each tumor in which the number of allelic losses or imbalances per tumor was divided by the number of informative loci per tumor. There was no association between the presence

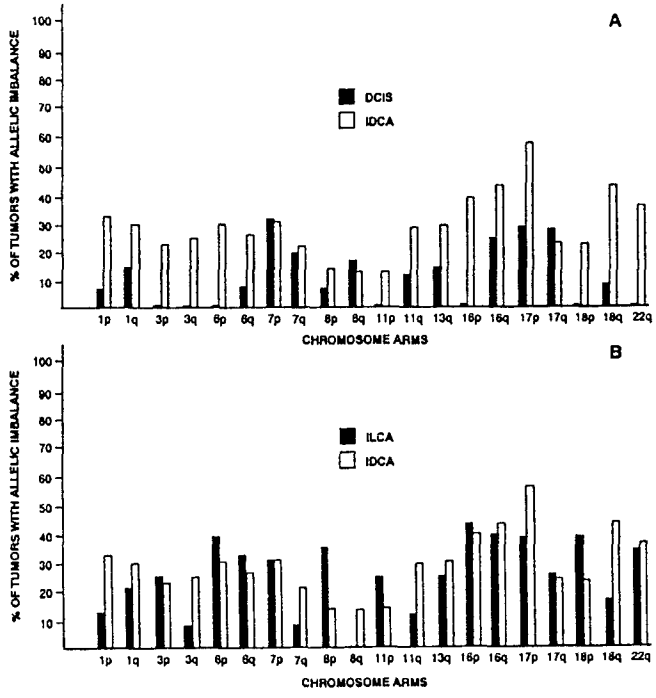

Fig. 1. *A*, comparative allelotype of breast DCIS (n = 23) *versus* invasive ductal carcinomas (*IDCA*; n = 29). *B*, comparative allelotype of IDCA (n = 29) with that of invasive lobular carcinomas (*ILCA*; n = 23).

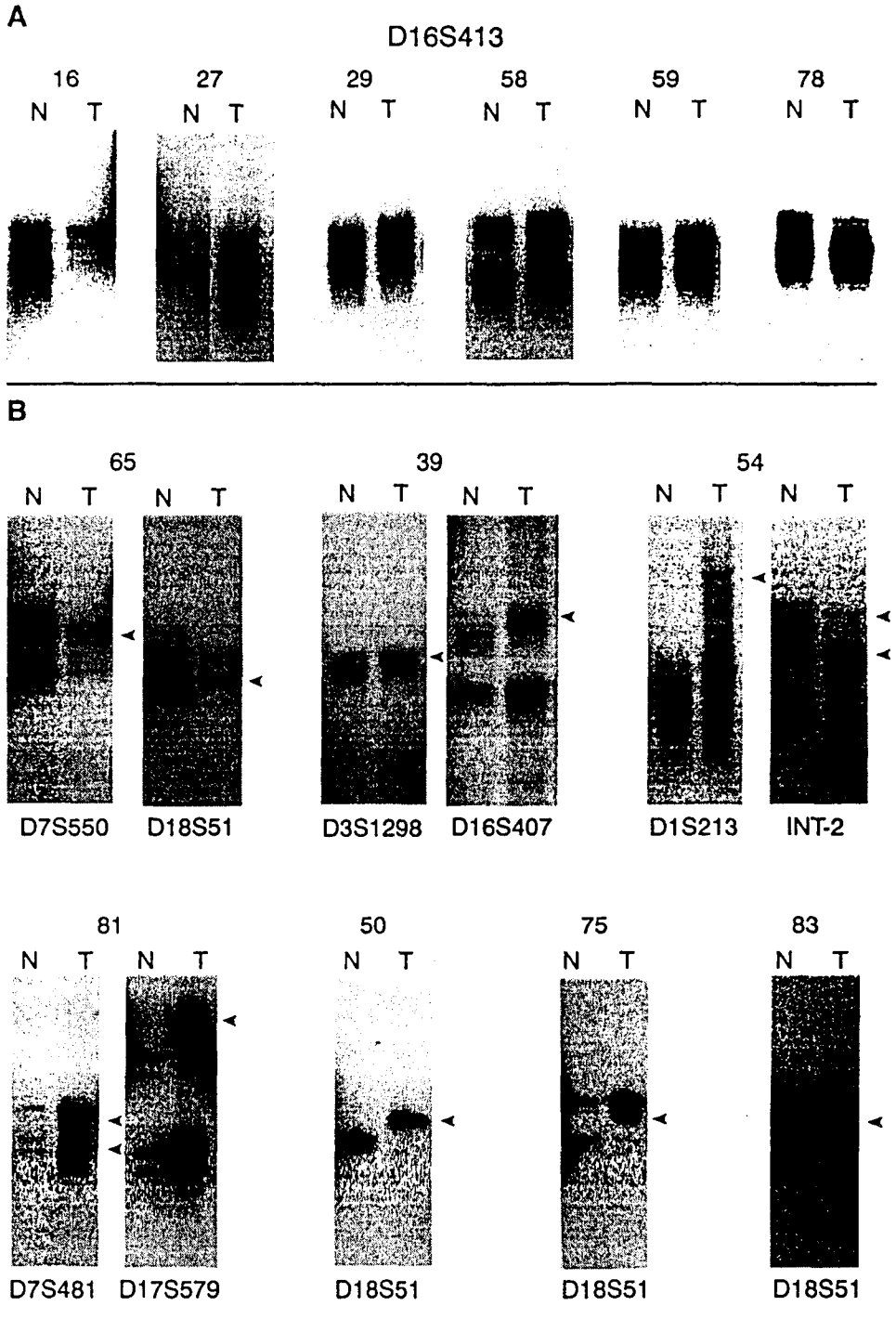

Fig. 2. *A*, representative microsatellite length polymorphism analysis of marker *D16S413* of paired normal (*N*) and breast tumor (*T*) samples obtained from microdissected paraffin-embedded tissue sections. Allelic loss or imbalance was frequently observed affecting this microsatellite marker. Samples 16, 27, 29, and 78 are from invasive ductal carcinomas, and samples 58 and 59 are from DCIS lesions. *B*, various representative breast cancer samples with RER+ phenotype (microsatellite instability). Note the abnormalities in allele size (*arrows*) in samples from the same tumors at different chromosome loci. Sample 65 is from a DCIS, sample 39 from an invasive ductal carcinoma, and the rest of the samples are from representative invasive lobular carcinomas. Marker *D18S51*, the only tetranucleotide repeat of the panel used, was frequently affected.

or absence of necrosis and the allelic loss index. However, a possible association was observed between allelic imbalance index and nuclear grade; lesions classified as non-high-grade (moderate and well differentiated lesions) had overall the lowest indices, with a few exceptions. All six DCIS cases without allelic abnormalities were classified as non-high nuclear grade. All the high-grade DCISs (poorly differentiated tumors) had indices higher than 0.10 (*i.e.*, they had loss or imbalance in at least 10% of the informative markers analyzed). The mean allelic imbalance index for the high nuclear grade DCIS tumors (n = 14) was 0.175 (± 0.06) and for the non-high grade lesions (n = 9) 0.095 (± 0.12). Although this putative correlation did not reach statistical significance, probably due to the sample size, it appears that there is a tendency for association between high nuclear grade and higher frequency of allelic losses and imbalances. This is in agreement with previous histopathological studies that indicated that high nuclear grade appears to identify subsets of DCIS with worse prognosis (22).

Invasive Lobular Carcinoma Allelotype and Microsatellite Instability. We also compared the allelotypic profiles of invasive ductal carcinomas with invasive lobular carcinomas. "Ductal" and "lobular" do not denote a different site of origin; in fact, it has been shown that most of both types of tumors originate in the terminal duct lobular unit (23). However, there are distinct morphological differences between the two histological types. Approximately 10–15% of all breast cancers are invasive lobular carcinoma (23). Histologically, lobular carcinomas have a distinctive infiltrative growth pattern with characteristic cytological features (23). As recently reviewed by Silverstein *et al.* (24), when compared with infiltrating duct carcinoma, reports on

Table 2 *Analysis of breast tumors with microsatellite instability by histology*

Histology	RER+ tumors/total tumors
Ductal carcinoma *in situ*	3/23 (13 %)
Invasive ductal carcinoma	4/29[a] (14%) 7/52[b] (13%)
Invasive lobular carcinoma	9/23[c] (39%)

a *versus* c; $P = 0.036$, χ^2 test. b *versus* c; $P = 0.012$, χ^2 test.

the prognosis of infiltrating lobular carcinomas vary considerably. Because characteristically these tumors show diffuse growth pattern without a prominent mass, they are more difficult to detect and diagnose. It is interesting that patients with infiltrating lobular carcinomas were reported to have statistically significantly higher risk of subsequent development of contralateral breast carcinoma (24). It has also been observed that the metastatic pattern of infiltrating lobular carcinomas differs from that of invasive ductal carcinomas (25). In our comparative allelotyping of invasive ductal *versus* invasive lobular carcinomas, we observed that allelic losses and imbalances affecting chromosome arms 1p, 3q, 11q, and 18q were more frequent in invasive ductal than in invasive lobular breast cancers (Fig. 1). On the other hand, 8p losses or imbalances were observed in 36% of invasive lobular tumors but in only 14% of invasive ductal tumors (Fig. 1). However, these differences between the two tumor types are not statistically significant at the 0.05 level, and a larger sample is necessary to conclusively identify specific anomalies.

Nevertheless, in the course of the allelotyping studies we observed that numerous lobular tumor samples showed frequent abnormalities in the allele size migration in polyacrylamide gels when compared with the matched normal controls (Fig. 2*B*). Abnormalities in size of simple sequence nucleotide repeats is a phenomenon described as microsatellite instability (26). This phenomenon has been described as a characteristic of tumors from patients carrying the autosomal dominant predisposition to tumors of the colon and endometrium, known as hereditary nonpolyposis colon cancer (26). These studies led to the identification of a group of human DNA mismatch repair genes as the cause of such general genomic instability phenomenon. Germline mutations in either the *Escherichia coli mutS* homologue *hMSH2* or the *mutL* homologues *hMLH1*, *hPMS1*, and *hPMS2* have been found in different subsets of hereditary nonpolyposis colon cancer kindreds (27, 28). Microsatellite instability, also known as RER phenotype, has also been reported to occur at various frequencies in various sporadic neoplasias other than colon cancer, such as cancers of the endometrium (29), stomach (30), esophagus (31), bladder (32), and other tissues. Yee *et al.* (33) reported microsatellite instability in 20% of breast cancers. In some other studies, however, a very low frequency of microsatellite instability was detected in breast cancer (34, 35). These discrepancies may be due to the number of loci and the type of simple sequence repeats assayed. For instance, it has been suggested that tetranucleotide repeats are more sensitive to RERs than are dinucleotide repeats (36). Recently, Glebov *et al.* (37) observed that individuals with a family history of breast cancer and with *p53* mutations had a higher frequency of abnormalities of chromosome 17 loci.

In our study of unselected breast cancer cases and mostly dinucleotide repeat markers, we observed the RER+ phenotype in 16 of the 75 breast cancer samples (21%). This figure is similar to that reported by Yee *et al.* (33). It was interesting, however, that when analyzed by histological subtype, only 13% (7 of 52 tumors) of ductal tumors (DCIS plus invasive ductal tumors) showed the RER+ phenotype, in contrast to 39% (9 of 23) of infiltrating lobular breast carcinomas (Table 2). This difference is statistically significant by χ^2 analysis ($P = 0.012$). Furthermore, if we exclusively compare invasive ductal carcinomas with invasive lobular carcinomas, the difference is still significant ($P = 0.036$). To address whether the observed microsatellite instability could be simply the consequence of a more aggressive tumor phenotype, we plotted the allelic loss index for the DCISs and the invasive ductal and lobular tumors, identifying those samples that were RER+ (data not shown). We observed that the lobular breast carcinomas do not appear to represent a more advanced tumor stage because overall they had a similar level of allelic losses as the invasive ductal tumors. In addition, some tumors with very few losses (low indices) were already RER+, including three at the DCIS stage.

Our data suggest that invasive lobular breast carcinomas appear to arise by a mechanism of carcinogenesis different from that of ductal breast carcinomas and may constitute a possible different pathological entity. These findings also support previous observations of different clinical behaviors of lobular breast tumors and ductal tumors (23–25). As mentioned earlier, the diagnosis of lobular breast carcinoma has been associated with a higher risk for development of multifocal or subsequent contralateral breast cancer (23, 24). The possibility exists that some patients that develop lobular breast tumors could harbor or develop mutations in any of the DNA mismatch repairs genes in the mammary epithelium, thus producing a field defect and constituting a facilitating event for the development of secondary mutations leading to tumor development.

Liu *et al.* (38) observed that only 1 of 10 patients with RER+ sporadic colorectal cancers had a detectable germline mutation in any of the known DNA mismatch repair genes, and most of the mutations found in the sporadic cases were somatic (38). It is important to analyze the role of microsatellite instability in breast cancer in light of the findings of Glebov *et al.* (37), who reported an association between microsatellite instability and familial history of breast cancer. The samples we used, however, were obtained at random from pathology files, and detailed information on familial history of breast cancer was not available for most of the cases, so we cannot evaluate in detail at this point the association between microsatellite instability and family history. Nevertheless, to at least partially address this point, we conducted telephone interviews with the patients (or their next of kin) who had breast tumors with the RER+ phenotype. We obtained detailed family histories on all first- and second-degree relatives (Table 3). Most of the breast cancers observed in family members, however, were among older relatives, suggesting that these are probably sporadic breast cancers. It was interesting that four of the nine patients with lobular breast cancer and the RER+ phenotype had, previously or synchronously, another breast cancer. In addition, three of these four cases were among those with multiple affected chromosomal loci. On the basis of our observations, we speculate that detection of microsatellite instability has the potential to be useful in identifying patients at risk of developing second breast cancers. However, these are only observations, and these findings will be certainly be substantiated with a larger data set.

In summary, in this report we identified for the first time the chromosome arms most frequently affected by losses and imbalances at preinvasive stages of breast carcinogenesis and those allelic loses involved in more advance stages of progression. In the course of these studies, we also observed that microsatellite instability was much more frequent in infiltrating lobular breast cancers than in ductal breast tumors. Our findings suggest that infiltrating lobular breast carcinoma is a different entity from ductal carcinoma and may arise by a different mechanism of carcinogenesis.

Table 3 *Breast cancer patients with microsatellite instability in their tumor*

Patient	No. of Loci Affected	Histology	Age	Other cancer[a]	No. of relatives with cancer: FDR[b] Breast	FDR[b] Other	SDR Breast	SDR Other
66	1	Ductal	54		0	2	0	1
5	1	Ductal	43		0	0	2	1
77	1	Ductal	63	Melanoma	0	1	0	2
8	1	Lobular	66	Breast	0	1	0	3
75	1	Lobular	46		0	0	0	0
65	2	Ductal	29		0	0	1	0
37	2	Ductal	66		2	0	1	0
80	3	Lobular	51	Breast, cervix	0	2	0	1
83	3	Lobular	63		0	1	0	2
24	>3	Ductal	49		0	1	1	4
39	>3	Ductal	52		0	0	1	1
50	>3	Lobular	71	Endometrium	0	0	1	3
51	>3	Lobular	73	Breast, endometrium	0	1	0	1
53	>3	Lobular	69	Breast	1	1	0	1
54	>3	Lobular	76		0	2	0	0
81	>3	Lobular	52		1	0	1	2

[a] Other neoplasia in the same patient.
[b] FDR, first-degree relatives (mother, father, siblings, children); SDR, second-degree relatives (aunts, uncles, grandparents, grandchildren).

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